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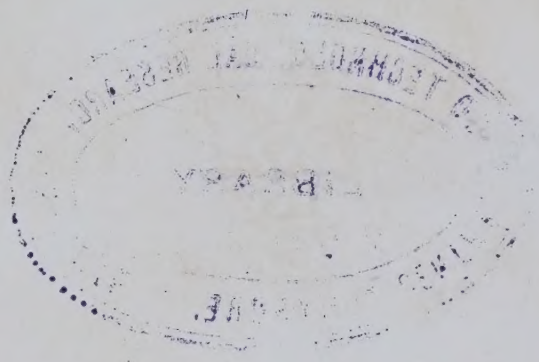
CHEMISTRY OF MUSCULAR CONTRACTION



CHEMISTRY OF

Muscular Contraction

Second Edition, Revised and Enlarged

BY

A. SZENT-GYÖRGYI
The Institute for Muscle Research at the Marine Biological Laboratory,
Woods Hole, Massachusetts

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Foreword

Up to 1939 the author regarded all his studies as a mere preparation for a more direct attack on life, which, then, was begun with muscle as material. The main result of the earliest work [*Enzymologia*, 9, 98–135 (1940)] was the realization that "life" and "muscle" are a complex problem and that a better understanding could only be achieved by a coordinated attack along different lines. This required a specially trained team, the organization of which was begun.

The results obtained from 1940–43 were published in the three volumes of "Studies from the Institute of Medical Chemistry, University of Szeged, Hungary" to be quoted briefly as "*Studies*." The discovery of actin, the "crystallization" of myosin, the discovery of actomyosin and its fascinating reactions with ATP were described in these pages. These observations are generally accepted now. Without them a comprehension of muscle would be impossible. It still gives the author a certain satisfaction that he printed these volumes himself, at the height of Nazism, in the English language, dedicating the first volume to the memory of a very noble-minded American, L. Kast, M.D., late president of the Josiah Macy Jr. Foundation, New York. Wishing to save these results from being lost and not expecting to survive himself, the author summed up the work in Supplement XXV of Vol. 9 (1945) of the *Physiologica Acta Scandinavica*. Publication in those pages was rendered possible by Swedish citizenship generously conferred upon the author. It is with profound gratitude that he recalls the help and shelter given to him by the Swedish legation, without which he could hardly have lived to see his paper appear.

After World War II, work was started on the ruins, as a small part of a wider plan: the building up a new and better world. Results were published in the newly founded *Hungarica Acta Physiologica* and summed up by the author in the first edition of this book in 1946 (referred to as "I"). These troubled years were little suited to quiet study. Some of the work had to be done in underground hiding places, and accordingly, it contained many weak points which could not be separated from the good ones. Consequently the author's book has met some very generous and some unduly harsh criticism.

In 1947–48 research, with the major part of the research group, was transferred to the United States. Here the weaker points of the earlier work were sifted out and new observations were made. The

glycerinated psoas fiber was introduced and allowed the study of contraction without relaxation, undisturbed by excitation. In association with R. W. G. Wyckoff, the mechanism of fiber formation was clarified, and the architecture of the contractile mass was outlined. Thermodynamics, which was but a shot in the dark before, has opened up a wide new avenue of approach. These observations warrant a new edition now. This present second edition is a blend of the earlier "Chemistry of Muscular Contraction" and "The Nature of Life" (Academic Press, New York, 1948).

That, from 1947–50, the work could be continued and that this book could be written are due to the support of The American Heart Association, which came to the author's help at an early stage and has generously supported him ever since. Much of the credit is also due to the generosity and understanding of the Public Health Service, Experimental Biology and Medicine Institute, Bethesda, Maryland, of which the author and some of his associates are special fellows. Thanks are due to the Viking Fund and its director P. Fejös for the encouragement and means which made it possible to follow up certain anthropological relations. Last, but not least, the author's thanks are due to The Institute for Advanced Study, Princeton, New Jersey, for the opportunity to write this book in its wonderful spiritual atmosphere.

Thanks are due to Mrs. P. Hajdu, who undertook the arduous task of translating this book from the author's English into its more traditional form.

Princeton, N.J. ALBERT SZENT-GYÖRGYI

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Abbreviations

ATP = Adenosine triphosphate

ADP = Adenosine diphosphate

AMP = Adenosine monophosphate

CP = Creatine phosphate

EM = Electron microscope or electron microscopic

EMP = Electron microphotograph

IP = Isoelectric point

ITP = Inosin triphosphate

MW = Molecular weight

Lines inserted on EMP's = 0.001 mm. If not stated otherwise all EMP's are palladium shadowed.

Chapter I

Introduction

1. Life and Organization

The biologist wants to understand "life," but gets into trouble as soon as he uses this word, since he is unable to tell what he means by it. He is unable to give a clear-cut definition, and pressed hard he will even have to confess that life, as such, does not exist at all. Nobody has seen life without matter. Thus it is not a thing in itself: it is a quality, a quality of systems of matter, which do curious things, mostly the opposite of what the physicist would expect them to do. We cannot separate life from matter any more than we can separate a smile from the lips.

Not everybody fares as badly as the biologist. The judge will not hesitate to call a man dead after he has been shot through the head and will not become confused when I explain to him that only the most recent and superficial evolutionary layer was blown off and that plenty of life was left below—the heart still beating, muscles twitching, and fermentation going on. The judge knows that the fellow will not love, or hate, sit, or walk any more; the system, having lost its long-range stability, is dead for all practical purposes. Instinctively, the judge knows that life is not an additive property and is more than the sum of single functions, as the individual is more than the sum of his parts and the stability of the system depends on the integrity of its pattern, the harmonious interaction of all its parts and functions, and once this harmony has been destroyed, the system must disintegrate.

Love, hate, and fermentation are all equally signs of life. The difference between these phenomena is only that being themselves of different complexity they involve structures of different complexity: while feeling demands the whole intact structure of the brain, fermentation can be performed even in a homogeneous solution—it is a question of the level of "organization."

By "organization" we mean that the thing is put together in a certain

fashion so that the whole is more than the sum of its single parts and has new properties of its own. "Organization" is the key to biology, and so we had better discuss it first in a simpler organism, say a watch. If we start building a watch from iron atoms, we must put these Fe atoms together in a certain fashion to make a piece of metal. This piece of metal has properties of its own, such as electric conductivity, which is not a property of the single Fe atoms. Not only has our piece of iron qualities of its own, which can no longer be described in terms of atoms (but only in terms of a piece of iron), but the single atoms themselves have also acquired new qualities by being put together into that system, and they themselves can no longer be described in terms of atoms. This same story repeats itself over and over again when we make cogwheels and levers out of that piece of iron, and when we put these wheels and levers together to make a watch, which can be described with reasonable simplicity only in terms of a watch and not as wheels, metals, or atoms.[1] If we try to analyze and understand this watch as scientists, our problem is not to give a transcendental definition of a watch. Our problem can only be to find out how it is put together to behave as a watch. In doing so we come into line with modern science, which has given up asking what things are, knowing that there is no means of understanding that anyway. Science contents itself by asking how things are put together to behave as they do, not "What is their substance?" but "What is their pattern or pattern of patterns?" —this is its problem.

Before changing over from my watch to a living system I would like to bring out one more point. There was no violation of first principles in the construction of our watch, which involved only three stages of organization: from atoms to metal, from metal to wheels, and from wheels to watch. This is a very low level of organization compared to living systems. Nevertheless the watch already begins to show some of that paradoxical behavior so characteristic of living systems, which, apparently, do not obey physics. Physical systems have their greatest stability at the maximum of entropy and the minimum of free energy where they are at equilibrium. Increase in entropy, equilibrium, and minimum of free energy mean "death" to my watch. According to physics my watch should increase stability when I drop it, which is contrary to my experience. As in living systems, maxima of stability do not correspond to energy-minima but to the patterns which allow the most harmonious interaction of all parts. Naturally, with

[1] We could also describe the watch starting from first principles, Fe atoms and coordinates X,Y,Z, but would soon arrive at complexities similar to those with which atomic physics is struggling.

its low degree of organization my watch will not duplicate all the properties of living systems, which have at least half a dozen further stages of organization. For instance, it will not wind itself up by releasing chemical energy. It will be worn out by use, as inanimate physical systems are, whereas living systems are worn out by inactivity and developed by use. My watch will tend to reach its free-energy minimum, whereas living systems have a strong tendency toward finding their own specific stability, that is, the pattern which allows the most harmonious interaction between the single parts of the system and between the system and its surroundings.[2]

When analyzing life, we cannot proceed as we did with our watch, and build it up, because we are unable to do so. The biologist can only proceed in the opposite direction and pull the system down gradually, piece by piece, looking around at every step and correlating function with the level of organization. We can expect to find life the richest and most colorful at the highest levels of organization. Color and functions will gradually become poorer as we descend along the scale of organization, fading out altogether at atomic levels where the word "life" has no meaning whatsoever.

Every level or organization will offer new vistas and will be equally thrilling to the researcher. We might start at the atomic level and observe what new qualities are formed when atoms join to form amino acids or nucleotides, when amino acid or nucleotides join to form proteins or nucleic acid, protein and nucleic acid join to form nucleoprotein, etc. We might also ask what new qualities appear when brain cells join to form a brain, or individuals to form a mob or a society, or plants and animals join to make a jungle.[3]

2. Muscle

The physicist or chemist, attracted by the simpler interactions, will prefer the lower or lowest levels of organization as material for study. If confronted with a more complex system, such as a higher living organism, he will tend to pull the system down into its simpler elements in order to learn how these are put together to do what they do. His quest is perfectly legitimate, and muscle will offer him a unique material for his study. What makes muscle unique is its mechanical

[2] Only certain patterns provide for such a state. This perhaps explains why evolution is discontinuous and why the egg cell, from which I developed, had to pass forms and patterns similar to those which my whole race had to pass in the course of its phylogenesis.

[3] Properties and reactions may even be antagonistic at subsequent levels or organizations, as in the case of the human individual as such or as a member of a mob or a political organization, as has been amply demonstrated by current history.

function involving fast and extensive changes. The rate of motion demands a simple geometrical structure. The wing muscle of the housefly, for instance, describes three hundred full cycles of contraction per second. Such rapid motion is possible only if the system is fitted together from small units, arranged with a high degree of regularity and held together, at least in certain directions, by weak forces only. This opens the possibility of taking the whole system to pieces without applying drastic methods. The dimensions of our muscles are in the range of an inch, and it will be shown in this book that it is actually possible to take the muscle to pieces, step by step, down to the dimensions of 10^{-7} inch (which is already a molecular dimension), studying at every step what the system is still capable of doing and coordinating function with the level of organization. More than that: not only can we pull the system to pieces, but having secured its pieces undamaged, we can put them together again and observe how the biological functions gradually reappear. So we can work up and down the scale of organization. Naturally we will be unable to restore the complete biomolecular architecture with its atomic precision, but we will be able to go some distance, making more complex systems out of the simpler ones, producing higher biological functions not possessed by the simpler systems and creating life, if we like to put it that way. There is no other living system known at present which offers a similar chance to the researcher.

The changes in muscle are not only fast, they are also extensive and violent. The wing muscle of the fly has to perform enormous amounts of work to keep that relatively heavy body afloat and give it its acceleration. This entails enormous changes in free energy and corresponding chemical changes. If science is the art of measuring, it will be the more successful the more there is to measure, and changes in muscle are almost explosive compared to the weaker and more uniform slow functions of other organs. Moreover, these changes are connected with changes in tension and dimension which can be registered by simple means or even observed with the naked eye.

Thus not only can we pull the system to pieces but we can also expect to find something to measure all the way through, down to the lowest levels.

Muscle holds out one more important promise, also dependent on its mobility. As far as we know, all living structures are built of the two basic constituents: proteins and nucleic acids, substances belonging to widely different chemical groups and having widely different properties. The fact that a living structure is always a combination of these two, a nucleoprotein, indicates beyond doubt that some sort of interaction between the two belongs to the most basic features of "life."

Nucleic acids consist of giant fibrous molecules, built of a great number of smaller units, so-called nucleotides. There is little hope of being able to analyze the interaction of such giant structures as a nucleic acid and a protein molecule. Motility in muscle excludes the presence of giant fibers which would interfere with motility, and so, in the contractile matter of muscle, instead of the nucleic acids themselves we can expect to find their smaller building stones, the nucleotides, reacting with relatively simple units of protein in stoichiometric proportions. This may open the doors to one of Nature's most important and at the same time most closely guarded secrets.

A few words about proteins in general may not be out of place. Owing to the complex geometry of non-motile organs it was hitherto impossible to decompose them without damaging, "denaturing," their units. So biochemistry has limited itself to the study of easily extractible, soluble proteins, which are extractible just because they do not constitute part of the structure, are spherical in shape, and are devoid of the forces which link particles together. The structure, the living edifice of the cell itself, constituted a rather unpleasant problem which was solved by calling it "residue" and sending it down the sink. What is called "protein chemistry" is to a great extent only the chemistry of non-structure-forming, easily extractible globular proteins, and so we must not be surprised if our structural proteins, isolated from the contractile matter, exhibit quite extraordinary properties when judged by the standards of current "protein chemistry."

3. A Little Philosophy and Methodology

If muscle is really such an extraordinary material and its mechanical function so unique, we might ask whether it will tell us anything about life in general, anything that will help us to understand other living systems. Will it help, for instance, to understand nervous action if we know how a muscle lifts weight?

Four decades of research have left no doubt in the author's mind that there is only one life and one living matter, however different its structures, colorful its functions, and varied its appearance. We are all but recent leaves on the same old tree of life and even if this life has adapted itself to new functions and conditions, it uses the same old basic principles over and over again. There is no real difference between the grass and he who mows it. The muscles which move the mower need the very same two substances for their motion as the grass needs for its growth, potassium and phosphate, the two substances we put on our lawn as fertilizer so as to have something to mow—a strikingly simple demonstration of the basic unity of living Nature. So, in principle, it does not matter which material we chose for our study of life,

be it grass or muscle, virus or brain. If we only dig deep enough we always arrive at the center, the basic principles on which life was built and due to which it still goes on.

Having thus chosen muscle as material for the practical advantages it offers, we have to make up our mind about three methodical questions: approach, method, and attitude.

Broadly speaking there are two approaches: morphology and physiology—structure and function. The two are inseparable and are really one and the same thing. Once the structure is there, it has to do what it does, and there is no function without an adequate structure. Moreover, muscle, like all other machines, must have two factors: an energy-yielding reaction and the specific structure in which this energy has to be liberated in order to do the specific work of that machine. The first can be expected to be identical in all cells, while the second may be better adjusted to the specific job.

As to the method and instrument of analysis, our problem is so involved, has so many sides to it, that if we want to achieve understanding we have to explore all lanes, use all methods of approach and all instruments available, in one single coordinated attack. There is one instrument, however, the use of which I would especially advocate, one which has lately fallen into disrepute, our eyes. It is definitely not incompatible with the dignity of a scientist to use them instead of limiting himself to pointer readings. Direct and close observation of the living material may still lead to important clues. If the author ever achieved any success in research it was due to petty observations.

As to attitude: we can take the wholistic attitude of the physiologist to whom the "muscle" and its "twitch" are units which cannot be disintegrated, or we can take the destructive attitude of the biochemist who feels happy only after he has pulled the living edifice down into molecules. It is equally difficult to understand the engine of a car without opening its hood, as it is to understand this mechanism from the heap of smallest parts into which it can be disintegrated. Fortunately, there is a third way: to start with the whole, keep it together for as long as possible, learning all we can about it. Then start pulling it to pieces, bit by bit, trying after every step to put it together again, to correlate the new partial system with the whole. This is what we will try to do.

4. Medical Aspect

For the medically minded biologist, muscle has one more great fascination, its medical importance. Human happiness and efficiency are dependent, to a great extent on the good working order of our

muscles, and no end of suffering is due to their disfunction. A slight contraction of the muscle of the artery wall may send up the blood pressure (hypertension) or may close down the oxygen supply (angina pectoris, vasospastic gangrenes), while defective relaxation of the skeletal body muscles (myotonia) or smooth muscle (dysmenorrhea, spasms) may cause trouble. For the major half of mankind the failure of muscle (heart) is the immediate cause of death, one which takes the horrid toll of one life per minute in the United States alone. Knowledge of these conditions demands a thorough analysis. It is childish to hope to be able to repair a machine whose structure and function are unknown, and the depth of our ignorance in this field is shocking. Large-scale reduction of human suffering can result only from the extension of our basic knowledge, from truth searched out for truth's sake, from which practical progress must flow as a natural consequence.

We can hope that a thorough understanding of the living state will help to develop a more scientific biology and medicine, while a more intimate knowledge of muscle will lay the foundation for a more scientific cardiovascular medicine. The A's (the angstroms) in which we express equilibrium distances of particles in muscle have their equivalent in human suffering. A better knowledge of these subtle equilibria will shift another equally subtle equilibrium, that of human happiness and suffering.

The Structure of Muscle

Muscle is a tissue that generates motion. There are many sorts of motion and correspondingly there are different sorts of muscle. There is, for instance, the rapid voluntary movement, such as that of the wing muscle of the housefly with its three hundred full cycles per second, and there is the muscle of the clam which contracts once and may keep the shell closed for the rest of the week. Other muscles work by producing rhythmic involuntary movements or light changes in their "tone." The muscle of the pregnant uterus may be dormant for months, to enter into violent activity at the end of its term, and relapse into inactivity immediately after delivery. The slower autonomous movements are performed mostly by the so-called smooth muscles, which consist of spindle-shaped cells dispersed in our organs and blood vessels, while rapid voluntary movements are performed by the impressive mass of "cross-striated" muscles (Fig. 1), which will be the main object of our present studies.

In deciding to work on cross-striated vertebrate muscle we have not defined our material yet, for there are considerable physical and chemical differences even between various muscles of one and the same animal. I can easily tell a rabbit from a chicken from its taste (in roasted condition) and even identify the single muscles of a chicken. If the muscles can be distinguished by such a crude and inadequate method, the differences in their composition must be considerable.

Equally important are geometric relations. The gastrocnemius muscle, for instance, has a featherlike structure, the fibers branching out from a central tendon. Such structure would make the calculation of energy changes very difficult. What we need is a muscle with long fibers running parallel, possibly with little connective tissue. It is easy to find the muscle which contains the least connective matter, for it is the quantity of connective tissue which decides the culinary value of a muscle, to which it is inversely proportional. The culinary value is expressed numerically on the right side of any menu. If this

FIG. 1. From A. Vesalius, humani corporis Fabrica. Brussels, 1543.

column of numbers is studied, a significant difference is always found at the level of filet mignon, scientifically: *Musculus psoas*. The reason is easy to understand. The contractile matter is a semi-solid gel which could easily be damaged mechanically. The protection it needs depends on its location. If much exposed, as is the case in muscles of the extremities, it is embedded into heavy sheets of connective tissues, the fasciae. The most protected major muscle of the body is the *Musculus psoas* lying in the depth of the abdominal cavity, protected from the back by the vertebral column and in front by the varying mass of the abdomen.

Luckily for us, the psoas, at the same time, is built of parallel fibers running continuously from one end of the muscle to the other. Owing to the paucity of connective tissue, these fibers can easily be separated into smaller bundles or even single fibers. A medium-sized

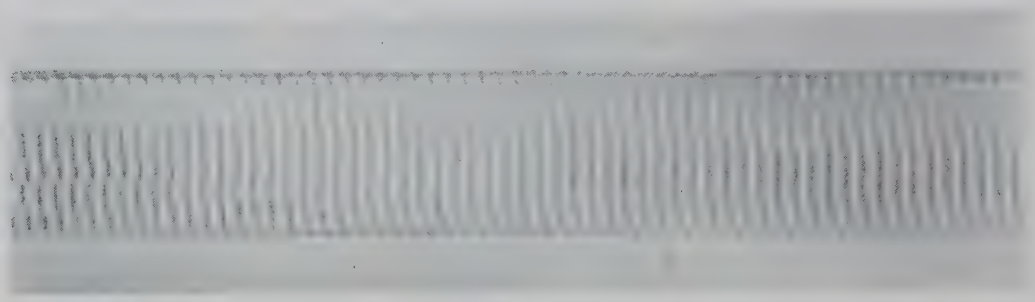


FIG. 2. Isolated muscle fiber in 50% glycerol. (Photo G. Matoltsy.)

rabbit will yield strips of parallel fibers 8–12 cm long, and it will not be difficult to secure single fibers even several centimeters long. Since the rabbit is easily obtainable, does not bite or bark, is neither too big nor too small, is rather cheap and very stupid, we will choose its *Musculus psoas* as the object of our study. This muscle provides a wonderful and unique material and, if not specifically stated otherwise, all our pictures and experiences relate to it.

The psoas of the rabbit is built of long and slender fibers, about $\frac{1}{20}$ mm wide, just visible with the naked eye, running continuously from one end of the muscle to the other (Fig. 2). Essentially, such a muscle fiber is a mass of contractile matter enclosed by a skin and is comparable to a sausage. The skin, the *sarcolemma*, is very thin. In the EM (Fig. 3) it appears as a very thin structureless membrane. According to physiology, the impulse coming from the nerve end is propagated by this membrane which separates the specific inner ionic atmosphere of the fiber from that of its surroundings. The impulse traveling along the sarcolemma is accompanied by a disturbance of the ionic balance which gives rise to the "action potential."

All tissues have a cellular origin and the muscle fiber is no exception. It is built of cells which merge at an early stage of its development. The fiber is their collective, a *syncitium* in which, as in other collectives, individuality is lost. The cellular origin is revealed by the single-cell nuclei, lying flat against the sarcolemma, surrounded by a small quantity of sarcoplasm.

Observed under the light microscope the muscle fibers give the impression of having a longitudinal striation which cannot be resolved.

FIG. 3. EMP of sarcolemma. (Rozsa, Szent-Györgyi, and Wyckoff, unpublished.)

Much more definite is a cross striation, a periodic variation of the physical properties along the axis.

The cylindrical space enclosed by the sarcolemma is divided into partitions by the thin Z-membranes, as a house is divided into stories. In mammalian muscle these Z-membranes are 0.002 mm apart, so that the fiber, which is about 0.05 mm thick, is divided by these membranes into rather flat, disklike partitions or *sarcomers*. The sarcomer is 0.002 mm high and twenty-five times as wide and resembles a one-

cent coin in shape, the whole fiber being comparable to a roll of such coins (Fig. 4).

The Z-membranes, though thin, seem to be built of rather solid stuff (Pease and Baker, 1949); their function is not known. Certainly, they must lend stability to the whole structure and insure that damage done to the contractile matter will remain localized in the sarcomer and not put the whole fiber out of commission. The Z-membranes may also be involved in the transmission of the impulse from the sarcolemma to the contractile matter, though there is no evidence available yet to this effect. The spacing of these membranes (and with it the height of the sarcomer) depends on the rate of movement. As shown by W. Szekessy,[1] the distance between Z-membranes is small

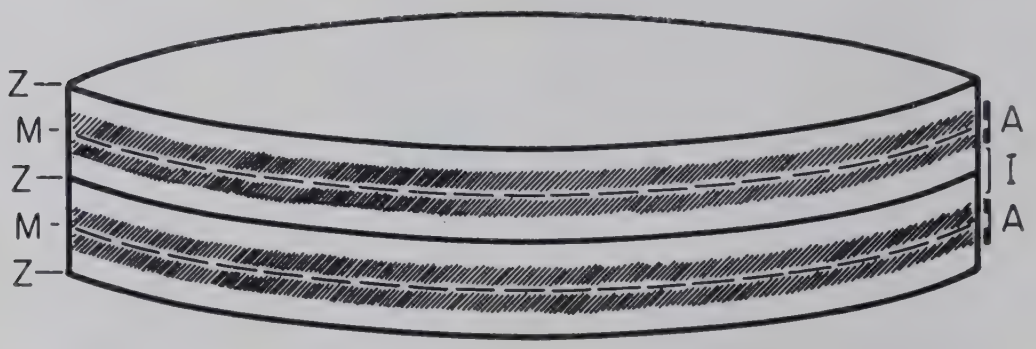


FIG. 4. Schematic picture of two sarcomers.

(0.002 mm), sarcomers are flat in rapidly moving muscles as, for instance, wing muscles of flying insects such as bees and frog or rabbit muscle, while in slowly moving muscles, such as those of *Limulus*, the thoracic or mandibular muscles of non-flying insects, the distance may be six times greater and reach 0.012 mm.

The matter contained in the fiber has a lower density and appears lighter in the microscope on both sides of the Z-membranes, seems to be denser, and appears darker toward the middle of the sarcomer (Fig. 4). If plain light is exchanged for polarized light and the muscle being examined is between crossed Nicolls, the picture turns round and the middle parts, being anisotropic, seem lighter, while the vicinity of the Z-membrane, being isotropic, appears dark. This isotropic disk (with the Z-membrane in the middle) is called the I-band, with the I standing for isotropic. The denser middle part is called the Q- or A-disk or band, with the A standing for anisotropic. On closer examination this disk is also found to have a very thin, membranelike formation in the middle, with a very thin sheet of looser material on both sides

[1] See I, pp. 90–91.

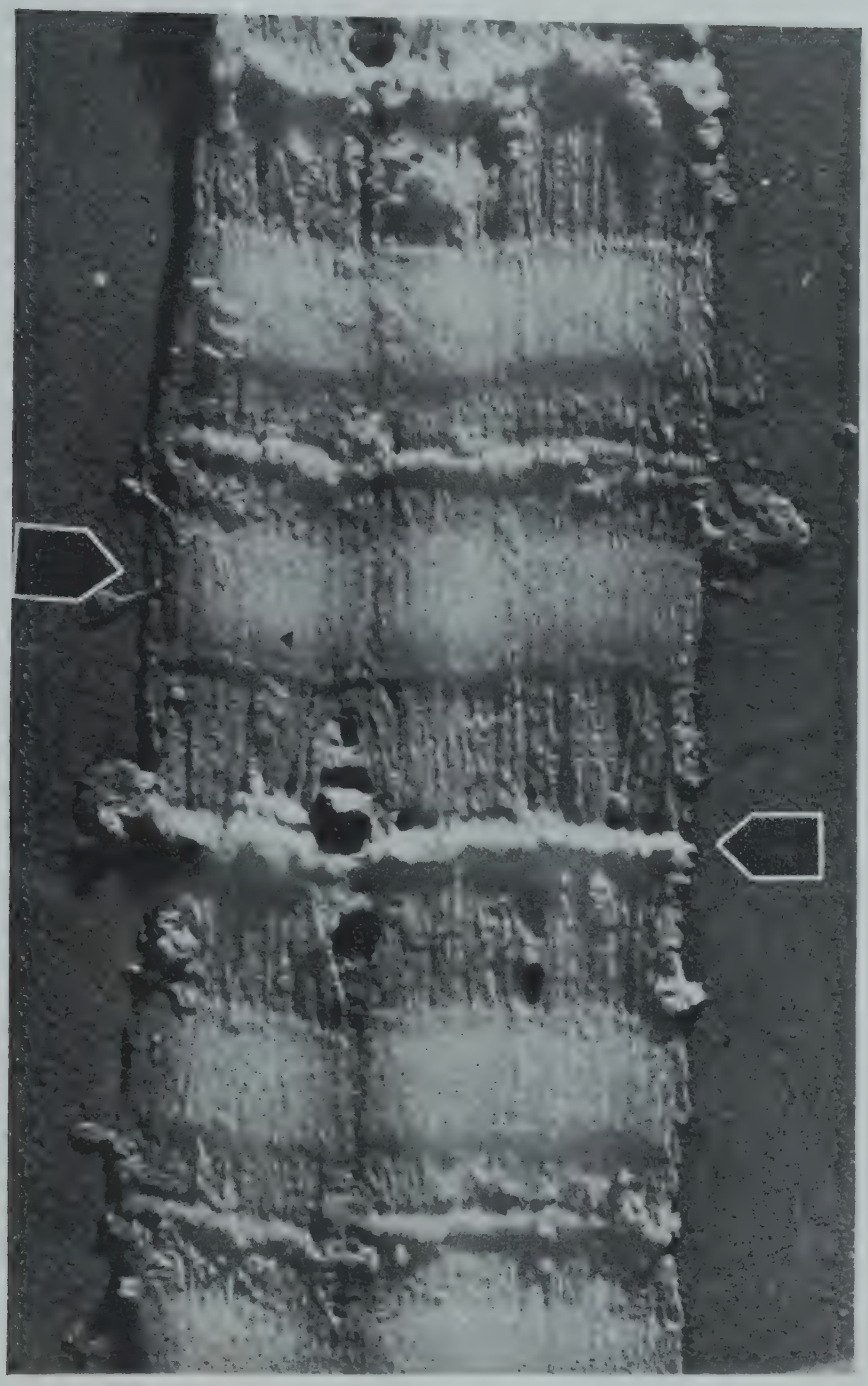


FIG. 5. EMP of a "fibril," spread on collodion. Black arrow on right marks a Z-membrane. (The next Z-membrane is just below the top margin of the picture. The mutual distance of the two Z's is 0.002 mm.) The left arrow marks an A-band. (Rozsa, Szent-Györgyi, and Wyckoff, 1950.)

(Fig. 4). This membrane is termed the M-membrane, since the membrane is in the *middle* of the sarcomer.

The mass of contractile matter which fills the sarcolemma and has this periodic variation very easily breaks up lengthwise, parallel to

the axis, into thin columns which have been termed "fibrils" by the morphologists. As will be shown presently they are artifacts, and fibrils do not exist in the intact rabbit muscle. Such a fibril, spread out on a collodion membrane is shown in Fig. 5, as it appears in the EM. It shows the periodicity beautifully. A Z-membrane is marked by the right-hand-side arrow. It is seen in the middle of a looser segment, the

FIG. 6. EMP of longitudinal slice of muscle. (Morgan, Rozsa, Szent-Györgyi, and Wyckoff.)

I-disk, similarly to the other Z-membrane, lying just below the top margin of the picture. The two I-disks enclose the Q-disk. As the picture shows this Q-disk itself is composed of five different segments. In the middle is the M-membrane, limited on either side by a narrower and looser field, the H-disk (rather accentuated in this picture). This in turn is bordered by two broader bands which reveal their greater density by their elevation and the consequent lighter shade. They definitely seem to contain more matter than the I- and H-bands. It is only these segments which display the high optical anisotropy, and thus

the name "A-band" will be reserved for these segments while "Q" will denote the whole disk lying between the I-bands, composed of A, H, and M. The left arrow in Fig. 5 marks an A-band.

The spatial arrangement of particles can best be observed in the EM study of slices in which the steric relations remain undisturbed. This technique is rather difficult and is still in its infancy. It has been applied to muscle by Morgan, Rozsa, Wyckoff, and the author. One of these pictures is reproduced in Fig. 6. In this picture the fibrils are seen running northeast-southwest, and the lighter middle part is an A-disk. Outside this segment the structure is looser and has disintegrated. In analyzing the lighter middle part the reader's active cooperation is requested. Here we have reached molecular dimensions and are at the limits of resolution, on the fringes of human ability to

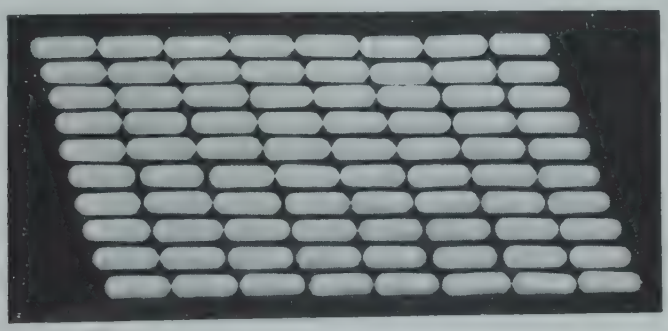


FIG. 7. Schematic representation of arrangement of particles in Fig. 6.

make things visible. At the moment of writing the author does not know how well the printer will be able to reproduce the subtleties of the very beautiful microphotographs. The author hopes that the reader, on closer examination, will be able to convince himself that the whole structure consists of uniform, slightly elongated particles, "*ovoids*" (ellipsoids of rotation), standing in a regular array as shown schematically and greatly magnified in the model in Fig. 7. The particles are arranged with great regularity, standing in rows both cross- and lengthwise, that is, associated both end to end and side to side.

It is a dramatic experience indeed to be able to *see* particles of molecular dimensions even if their contour is not very distinct. In fact if they lay isolated, the particles could not be seen at all, they could not be distinguished from the granulation of the background, the membrane on which the material is spread. What is shown in Fig. 6 is less the particles themselves than the symmetry planes of their regular two-dimensional array. These planes enable us to make an approximate calculation of the molecular weight of the particles. The distance between subsequent planes is 100 A crosswise and 300 A lengthwise, enclosing a space of $100 \times 100 \times 300$ A^3 $= 3 \times 10^6$ A^3, which

FIG. 8. EMP of cross section of muscle. (Morgan, Rozsa, Szent-Györgyi, and Wyckoff.)

is the space occupied, though not completely filled, by one particle. With specific weight 1 this volume would correspond to a weight of 3×10^{-18} g; $6 \times 10^{23} \times 3 \times 10^{-18} = 18 \times 10^{5}$ g. Naturally, only part of this is protein which has a higher specific weight than 1. At any rate, we are in the dimension of MW 10^{6} g.

In a cross section these elongated particles must appear as round. Such a cross section is shown in Fig. 8. The reader studying the two fibrils in the middle, or the fibril with the big white spot, will discover that in this cross section the particles are also arranged with great regularity in a hexagonal packing, as shown schematically in Fig. 9. Every third unit in this last picture is made somewhat darker to show that in this arrangement every particle is surrounded by six others and gives the closest packing.

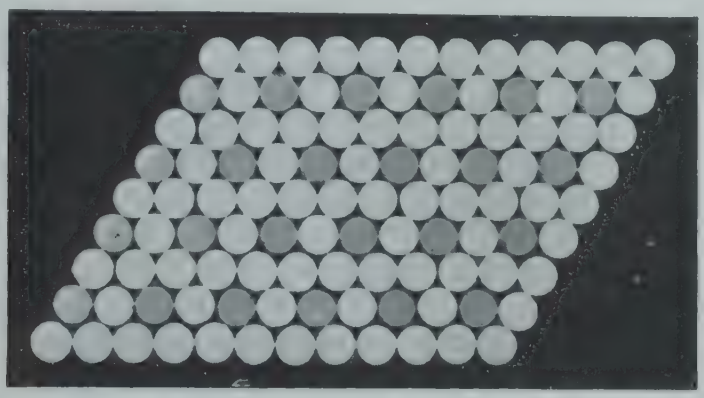


FIG. 9. Schematic representation of arrangement of particles in Fig. 8.

The regularity is thus a three-dimensional one and structures composed of units arranged with three-dimensional regularity are called crystals. Since the contractile matter which fills the fibril is a semi-solid gel, it would perhaps be somewhat misleading to call it simply a crystal, and the affix "liquid" might be justified. "Liquid crystals" or "tactoids," such as those of the tobacco mosaic virus, are usually built of elongated particles which are oriented and partly held together by their dipole forces, which, being stronger in the direction of the longer axis, induce a rather strong end-to-end association. As the muscle is exposed to strain along the axis, the end-to-end association of particles must be especially strong. *If* the positive and negative poles are arranged alternately, as shown schematically in Fig. 10, then these forces *may* also cause side-to-side association, crosswise, which will be much inferior in strength to the end-to-end one. Such a system will have a stronger tendency to disintegrate crosswise than lengthwise.

Figure 11 shows a fairly large field of a cross section of a muscle

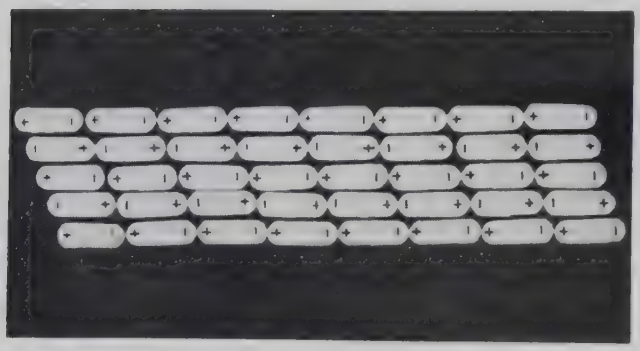


FIG. 10. Schematic representation of possible arrangement of dipoles.

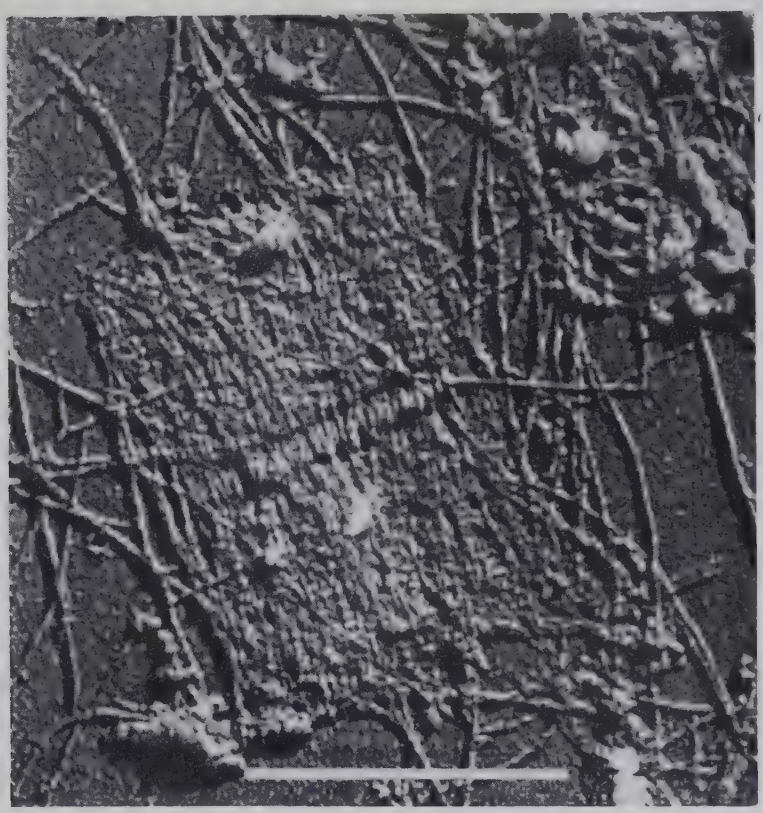
FIG. 11. EMP of cross section of muscle. (Morgan, Rozsa, Szent-Györgyi, and Wyckoff.)

fiber consisting of what one would think at first sight to be the cross section of fibrils. On closer examination, however, one will discover that all the narrow splits between fibrils tend to meet at 120°, which is the angle formed by the symmetry planes of a hexagonal array. This shows that these splits are but cleavage planes of the continuous crystalline mass of contractile matter. As the material shrinks, it breaks up along the cleavage planes of the crystal. The muscle fiber thus contains no preformed fibrils and the contractile matter forms one continuous mass. Fibrils are artifacts, owing their origin to the treatment, chemical or mechanical injury outside the body. If shrinkage is still stronger, the whole structure breaks up into completely separated columns with empty space between them devoid of matter, as is the case in Fig. 8.

If the muscle fiber is exposed to strong mechanical injury, as is the case in the Waring Blendor, the whole structure falls into single rows of particles, still held together along the axis. Such "monomolecular" threads are shown in Figs. 12 and 13. These fibers are about 100 A wide and thus correspond to one single row of the ovoids seen in the sections. In Fig. 13 the single fibers are still held together loosely so that one can recognize the M-membrane in the middle. The length of these fibers indicates that they mostly break at the Z-membrane and run continuously through the whole sarcomer from Z to Z. Ardenne and Weber were the first to observe threads of this dimension in muscle extracts and Hall, Jakus, and Schmitt (1946) were the first to see them in the muscle fibrils. They called them simply "filaments," and it was generally thought that as the muscle is a bundle of fibers, so the fiber is a bundle of fibrils, and the fibril a bundle of filaments. As we have seen, in reality there are neither fibrils nor filaments, both being but the remnants of a disintegrated three-dimensional structure, though in some preparations, such as that in Fig. 14, obtained by disintegrating the cross-striated muscle of *Limulus*, these "filaments" have a strong individual character.

Hall, Jakus, and Schmitt observed that their filaments had a beaded character. "The nodes are often almost equidistant along the filament and, in some regions of the fibril, they may be aligned laterally to produce a fine cross striation with a period of about 400 A in the direction of the fiber axis." Baer in his small angle x-ray pictures of muscle also described a periodicity of 400 A. Draper and Hodge in their incinerated slides found a periodicity of 400 A in the distribution of ashes and observed a cross striation of similar dimensions in their stained muscle preparations. The periodicity along the fiber

FIG. 12



FIGS. 12 and 13. EMP of filaments of blendored muscle. In Fig. 13 M-line is still visible. (Rozsa, Szent-Györgyi, and Wyckoff, 1950.)

axis in Fig. 6 is in the range of 300–400 A and so probably all these observations have a common structural basis.

So far we have considered only the content of the muscle fiber, but muscle contains one more rather important structural element, a rich net of peculiar fibers described by Rozsa, Wyckoff, and the author (1950), and shown in Fig. 15. This net surrounds the fibers, adhering to their surface if these are isolated. The single fibers are 250 A wide

FIG. 14. EMP of filaments of *Limulus* muscle. (Rozsa, Szent-Györgyi, and Wyckoff, unpublished.)

and thus could never be distinguished in the light microscope. They have a structure resembling money rolls (Fig. 16), being built of little disks, about 250 A wide and 200 A high. They have a tendency to unite to form continuous sheets, as shown in Fig. 17. They must contribute greatly to the mechanical stability of the muscle and protect the semi-solid contractile matter and the thin sarcolem against injury. According to their structure they can be expected to show limited "high elasticity." The elastic properties of muscle observed on stretching

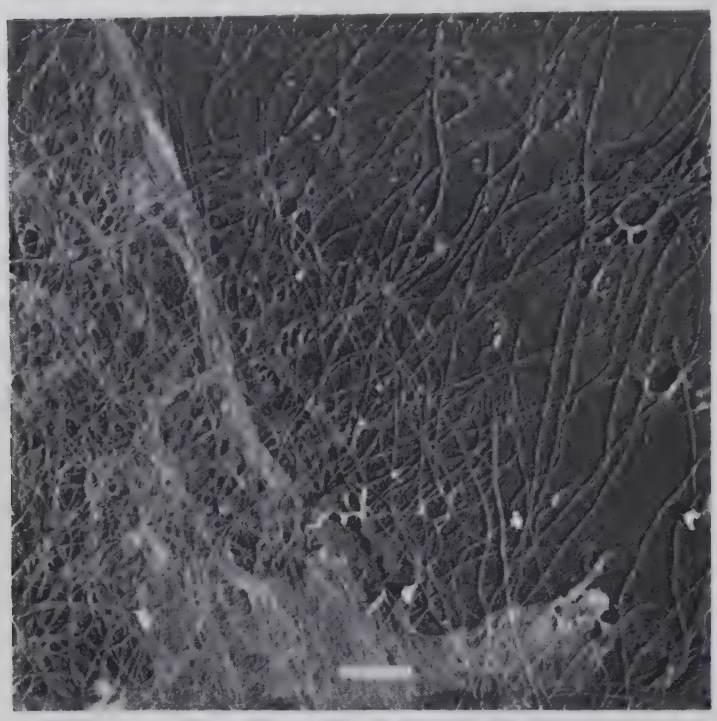


FIG. 15

FIG. 16

FIG. 17

FIGS. 15, 16, and 17. Fibers from the net surrounding the muscle fibers. (Rozsa, Szent-Györgyi, and Wyckoff, 1950.)

must be due to a great extent to these fibers.[2] According to its location, muscle may also contain varying amounts of typical connective tissue. If the muscle is located on the surface and exposed to injury, it may be enclosed into fasciae and divided by septa of connective material. Accordingly, Reed and Rudall found typical collagen fibers in their prep-

[2] Possibly it is due to this fiber net that on stretching muscle shows a rather complex thermoelastic behavior (Wöhlisch) which, perhaps, is responsible for the fact that experiments in this direction have not yet led to a comprehensive theory. Behavior on stretching must depend, apart from the contractile matter, on the sarcolem, on the fiber net described and finally on the regular connective material with its collagen fibers.

At 33 per cent stretch there is an important turning point in the reactions of muscle. It is at this point that the normal behavior of muscle gives room to the Weber paradox (E. Weber), thermoelastic inversion takes place (Wöhlisch), and it is about here that the stretch begins to be irreversible. Possibly this is the stretch at which the single ovoids seen in the EM come to lie too far to exert an appreciable attraction upon one another and the crystal breaks up lengthwise. Calculations based on this assumption and attributing the elastic modulus to these attractions lead to bond energies between ovoids of the dimension of 10,000 cal.

aration taken from more exposed frog leg muscles. The fibers described above seem to be closely related to collagen, and transitory forms are not too infrequent. The confluency of three particles 200 A in diameter would give the 600 A periodicity of collagen. Porter and Vanamee observed similar fibers in embryonic tissue cultures.[3]

On stretching, muscle must show a very complex behavior which must be governed to a great extent by these different fibers lying outside the sarcolemma. Contrary to this, we can expect that in contraction these fibers do not modify behavior of muscle which must be dominated by the changes in the contractile matter.

Readers more profoundly interested in morphology are referred to Barer's review which gives an admirably lucid picture of the obscurity of the subject.

[3] In muscle protected by a solid shield, as is the case in *Limulus* or *crustaceans*, even this fiber net seems to be missing or poorly developed, for even a soft thread, applied as ligature, easily cuts through the muscle.

Whole Muscle

In Chapter I we decided to disintegrate the muscle, but decided to do this gently, step by step. The gentlest thing we can do to it is to do nothing; just cut away the rabbit (or excise the muscle).

If the psoas of the freshly killed rabbit is excised, it shortens by about 20 per cent, which indicates that at rest the muscle was not really in equilibrium, but was under a strain, having been stretched from its "equilibrium length" to its "rest length." We need very little force to stretch the muscle from its equilibrium length to its rest length again, but as we reach the rest length, the force needed for further extension suddenly increases and becomes proportional to the extension, the muscle now obeying Hooke's law. From this point on the muscle can still be stretched by about 40 per cent and snaps back to its equilibrium length when released. The muscle thus shows limited "high" or "long-range" "rubberlike" elasticity. Rubber also demands very little force for its initial extension. Muscle is helped over this range (in which precise motion would be impossible) by being suspended in a somewhat stretched condition, which brings it into the Hooke region. If stretched beyond 40 per cent, resistance suddenly increases, slipping occurs, and the extension becomes more and more irreversible. Finally the muscle breaks.

If we wait a few, say four, hours after the death of the animal, we find an entirely different picture. When excised, the muscle does not shorten. If stretched, it offers maximum resistance at once. If stretched and released, it does not snap back, the stretching being entirely inelastic. The muscle can be stretched only a few per cent and then breaks. Its consistency also has changed from supple softness to rigidity. This was known to man in all ages as the accompaniment of death and is called *rigor mortis*, the stiffness of death. If the changes are measured by more accurate methods, they are found to set in immediately after death, to increase gradually, reaching maximum within a few hours. Only infrequently is there a short lag immediately after

death. Parallel to the increasing stiffness, myosin, the main structural protein of the fiber, becomes gradually more and more insoluble, as shown earlier by Saxl; Deuticke; and Weber and Meyer.

Erdös (1943), trying to find the chemical reaction responsible for these changes, measured the increasing stiffness and estimated simultaneously the concentration of ATP in muscle. ATP denotes a nucleotide built of adenine, pentose, and three phosphate groups coupled in a row by anhydrid links (Fig. 18). Erdös found that the two, stiffness and post mortem decomposition of ATP, go hand in hand, perfectly parallel. He was also able to show that the disappearance of ATP not merely accompanied rigor but was actually the cause of it, for by adding ATP he could render the myosin soluble again. It is easy to show also that the psoas fibers which are rendered inelastic by washing out their

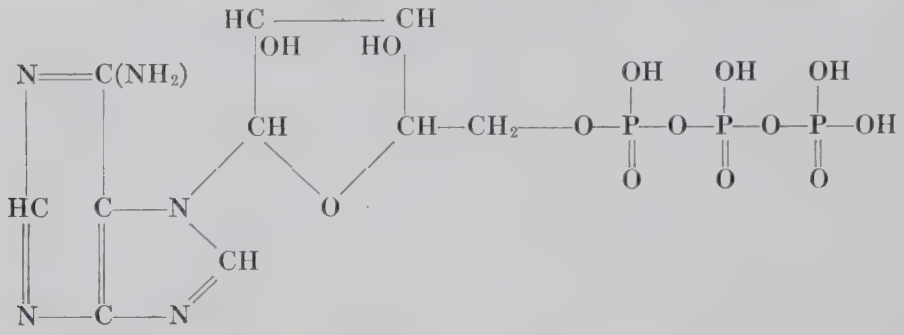


FIG. 18. ATP (adenosine triphosphate).

ATP can be rendered elastic again by restoring their nucleotide to them. Bate-Smith and Bendall corroborated and extended these results, while Borbiro found that parallel to the decomposition of ATP contractility also disappeared.[1]

These observations leave no doubt that the whole physical state and function of muscle depends on its ATP and that ATP, in resting muscle, is present in an active state and is in contact with the contractile matter.[2]

[1] The two curves of contractility and ATP concentration went parallel only until the ATP concentration fell to one-fourth its normal level. At this point rigor was fully developed and the muscle was not contractile at all. Unlike the other three-fourths, the decomposition of the one-fourth is exceedingly slow. This ATP seems to have a different localization and action than the other three-fourths. Borbiro also noted that excitability disappeared when the ATP concentration fell to one-half its normal level.

[2] This conclusion, that ATP is connected with the contractile matter, is in contradiction to Caspersson and Theorell's observation, according to which ATP, at rest, is limited to the I-band. This conclusion was arrived at by measuring the ultraviolet adsorption of the A- and I-bands. Matoltsy (personal communication) was able to corroborate Caspersson and Theorell's observations, but found that the ultraviolet

If the muscle is left alone, the rigor disappears spontaneously in a day or two. This is not due to the resynthesis of ATP but to the autolytic disintegration of the structure. At higher temperatures (45°C) the ATP-free muscle becomes elastic again, it "melts" even without denaturation of the proteins (the author, 1949).

The physical properties of muscle are thus not simply properties of the constituting proteins. The properties of these proteins depend to a great extent on their interaction with ATP which governs their properties, is involved in contraction and possibly also in excitation, and can thus justly be called "the master substance of muscle."

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adsorption was not altered by thorough washing which must have eliminated all the ATP. Evidently the ultraviolet adsorption measured by Caspersson was not due to ATP but to some other member of the nucleic acid group, probably a nucleoprotein.

The Physical Disintegration of Muscle

By "washing" the biochemist means suspending pieces of a tissue in water, expecting that the water will extract the crystalloids and water-soluble proteins while the structural proteins will be left behind. The psoas, too, can be washed. Instead of water we can use 50% glycerol of 0°C which conserves the structure better than water and allows us to keep the washed muscle at —20°C without freezing. The author kept strips of the psoas under these conditions for more than half a year without their losing their contractility.

By washing we thus decompose the muscle into two fractions, one being the structure and the other the soluble matter. Having done this we can try, according to our original plans, to put things together again and restore its extract to the extracted muscle. If interested in the crystalloids, instead of concentrating our wash water, we had better

Fig. 19. (*a*) Washed muscle fiber at rest length. (*b*) The same a few seconds after the addition of 0.2% ATP.

prepare a more concentrated extract directly by throwing the freshly minced muscle into an equal volume of boiling water and heating the system quickly to 80°C or so, putting it on a cheesecloth, and then pressing out the protein coagulum. The press juice (clarified by centrifugation or filtration) contains the crystalloids of the muscle in a relatively high concentration.

If an isolated fiber of the washed psoas is immersed in this juice, it contracts instantaneously, with great speed, to about one-third its rest length. This reaction is shown by the fiber bundle in Fig. 19. Within 2 to 3 minutes the muscle may even contract to one-fifth its original length. If a thin fiber bundle containing 4 to 6 fibers per

diameter is placed in the boiled juice and connected to the isometric lever, the instrument will indicate 1-2 kg tension per square centimeter, the tension developed by the same muscle *in vivo*. This leaves no doubt that *it was the normal mechanism of contraction which was put into action* by the juice.

The enzymic activity and temperature dependence of the function of such glycerinated fibers were lately the object of interesting studies by Korey, while A. Weber and H. H. Weber (1950) extended measurements to single fibers and obtained tensions up to 4 kg per square centimeter.

The first question put by any chemist would be: What is or what are the substances in the juice responsible for eliciting this contraction? A little scientific cookery gives the answer, showing that they are ions and ATP. So we can replace our boiled juice by a solution containing 0.1 *M* KCl (or NaCl), some $MgCl_2$ (0.001 *M*) and 0.2–0.3% ATP, as Na or K salt, in which concentration these substances are present in the boiled juice. The action of K is not specific and can be replaced by Na. Mg is more specific and cannot be replaced by Ca. ATP is entirely specific and, as far as we know, cannot be replaced by any other substance, except by a higher concentration of the closely related ITP (Lajtha, 1950; Bowen and Spicer 1950), which is nothing but ATP without its $6NH_2$ group. This leaves little doubt that *contraction* in *muscle is a reaction of the structure brought about by ATP and inorganic ions.*[1] This explains why Borbiro found contractility parallel to ATP concentration in muscle.

We can now go one step farther with our destructive work, roughly decomposing the extracted muscle by suspending it in water, putting it into the Waring Blendor and reducing it to a pulp. We have now exactly the same system as before, the only difference being in the dispersity of the insoluble proteins, the higher architecture having been destroyed and reduced to a suspension. This suspension, in the presence of 0.1 *M* KCl, tends to settle slowly to form a loose, voluminous, flocculent precipitate (S. C. Roy). As before, the addition of this very specific ATP will cause a striking change, but instead of contraction a violent precipitation occurs: our suspension precipitates violently, forming a dry-looking granular precipitate which settles quickly to the bottom of the test tube, occupying a small volume there (Roy). This excessive precipitation produced by ATP has been termed *superprecip-*

[1] Buchthal, Deutsch, Knappeis, and Petersen (personal communication) obtained contraction with free ATP in the absence of salts. This only shows that K or Na ions can be replaced by H^+. Banga and the author repeatedly emphasized that the action of K or Na in contraction of actomyosin is not specific.

itation to distinguish it from the weak precipitation caused by salts alone in the absence of ATP. Evidently this superprecipitation is essentially identical with the contraction seen before. It could be called "contraction without architecture."

Precipitation is a very common, everyday colloidal phenomenon due to a loss of charge with the consequent loss of repulsions and hydration. If this takes place within the molecular architecture of the fibrils, it leads to contraction. If superprecipitation is a "contraction without architecture," then "contraction" might as well be called "precipitation within that structure."

These childishly simple experiments, which actually should be repeated in every physiological class, have led us to three very interesting observations. One is that contraction is connected with major changes in charge, a discharge, or depolarization of electric forces. Another is that ions are involved in this reaction, as they are usually involved in colloidal reactions in which a discharge of particles takes place. The third, and perhaps the most important observation, is that ATP, the "master substance" of muscle, governs the physical properties of muscle not only at rest but is also instrumental in contraction. This experience can be supplemented by the results of physiology, which show that the terminal P—O—P link of this molecule, with 11,000 cal of free energy, is the source of the energy of muscular contraction.

What makes these observations the more remarkable is the fact that ATP is a nucleotide, one of those small bricks from which nucleic acids are built, and our hope that in the contractile matter we will find nucleotides instead of nucleic acid is realized.

Our experience also showed that ATP has two distinctly different actions. It makes the resting muscle supple and elastic—a static function, and makes the muscle contract—a dynamic action. Naturally, the first must also be involved in the second, for if the muscle were rigid it could not contract.

Chapter V

The Chemical Disintegration of Muscle

Kühne (1868) was the first to observe that a rather viscous globulin-like protein could be extracted from muscle by means of strong salt solutions. He termed it "myosin." The study of this new protein was taken up by Danilewsky, Halliburton, and v. Fürth, who corroborated and extended Kühne's experience. The existence of the new protein, myosin, became accepted, and it was assumed that it was a building stone in the contractile mechanism. Fürth's paper in 1895 concluded this period.

The study of myosin was taken up again by H. H. Weber three decades later (1925). Weber (1934) also devised a most ingenious method for the study of this protein: he squirted its salt solution into water in a thin jet. When the salt was dissolved, the myosin gelled in the form of threads. These threads are a most attractive experimental material, their charm being partly derived from their resemblance to the muscle fiber.

Soon Edsall and Muralt also joined in the investigation of this protein, paying special attention to its physical properties, in the first place to its double refraction of flow.

Interest turned toward myosin for the third time when Engelhardt and Ljubimowa (1939) suggested that myosin might be identical with the ATP-ase of muscle, the enzyme which splits the P-O-P link in ATP, releasing its energy, detaching the terminal phosphate as free inorganic PO_4. The idea that the contractile substance should act as the enzyme which liberates the energy needed for its function was most exciting. It promised a deeper understanding of energy coupling, i.e., the relation of the energy-liberating chemical process and the energy-consuming physical process, telling us how chemical energy can be converted to mechanical work in the animal body. Soon after (1941), Engelhardt, Ljubimowa, and Meitina (1941) gave another example of a very specific interaction between myosin and ATP, showing that myosin threads become more extensible under the influence of this nucleotide. These

instances of specific and rather intriguing interaction between myosin and ATP were further enriched the same year (1941) by Needham and his associates who showed that the viscosity of myosin, dissolved in 0.6 *M* KCl, dropped reversibly under the influence of ATP. Munch-Petersen showed that ATP caused strong expansion in monolayers of myosin, the reaction being highly specific.

Schramm and Weber (1942) discovered that "myosin" is not homogeneous and contains a "heavy" fraction which settles faster in the ultracentrifuge. In the EM this "heavy" myosin was found by v. Ardenne and Weber to consist of long threads of molecular dimensions.

The study of myosin was also taken up in 1939 by the author's laboratory. It was soon found (Banga and the author, 1941) that myosin solutions prepared by short extraction had a relatively low viscosity, while myosin solution prepared by protracted extraction had a high

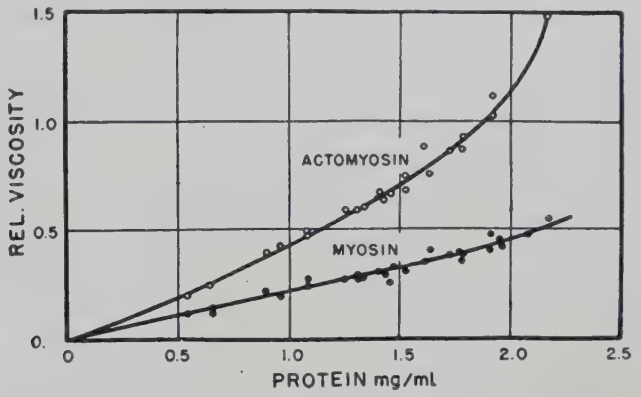


FIG. 20. Upper curve, viscosity of actomyosin extracted as such from muscle (myosin B); lower curve, the same after the addition of ATP. This curve is marked "myosin" being approximately identical with the curve of the myosin present (quoted from I).

viscosity without containing considerably more myosin. This highly viscous myosin was termed "myosin B" to distinguish it from the "myosin A" of low specific viscosity. The high viscosity of myosin B, dissolved in 0.6 *M* KCl, dropped to a low level on the addition of minute quantities of ATP (Fig. 20). This drop in viscosity was termed "activity" and gave a quantitative measure for the analysis of the myosin A-B transformation. This study was undertaken in the author's laboratory by F. B. Straub (1942), who showed that the gradual increase in viscosity was due to the dissolution of another protein from the minced muscle, a protein distinct from myosin. This protein was termed "actin." Actin united with myosin to form the highly viscous "actomyosin," which, in the presence of a high salt concentration, dissociates into its components again on the addition of minute quantities of ATP.

If no special precautions are taken, some actin always goes into solution along with myosin, greatly modifying the physical properties of the latter and in many instances the observed properties of myosin, as for instance its double refraction in 0.6 *M* KCl, were actually the properties of the actin or the actomyosin present, while the drop in viscosity observed by Needham *et al.* was evidently due to the dissociation of this complex.

This change of ideas could not fail to cause some confusion in the nomenclature. The "myosin" of earlier investigators was evidently an undefined mixture of myosin and actomyosin. The author's myosin-A was a myosin poor in actin, his myosin B an actomyosin containing one part actin to five parts myosin. Weber's "heavy myosin" is actomyosin; his "light myosin" is just myosin. Dubuisson's β-myosin is myosin; his α-myosin is actomyosin.

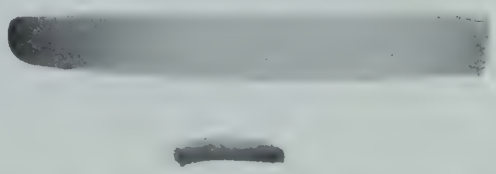


FIG. 21. (*a*) Actomyosin thread, prepared according to H. H. Weber. × 30. (*b*) The same a few minutes after the addition of ATP.

Myosin is a pleasant and generally accepted name, linked to the names of the pioneers in this field and so we should retain it. The author proposes to use myosin as the name of the actin-free myosin. Actin is actin and the complex of actin and myosin is actomyosin. The "myosin" of earlier investigators (undefined actomyosin) will be termed "myosin," that is myosin between quotation marks. Some authors, such as Astbury and his associates seem to be inclined to retain the name "myosin B" for the natural actomyosin, extracted as such from muscle, to distinguish it from the actomyosin obtained by mixing actin and myosin.

Actin and myosin both have remarkable properties, evidently connected with their special function, contraction, but none of them shows any signs of contractility. ATP, added to their solution in the same ionic milieu in which it elicited contraction in washed muscle, interacts with both of them, producing changes in their physical state, but these changes are in no way similar to contraction and superprecipitation.

If ATP is added to actomyosin, a dramatic change occurs, which in many ways is similar to that observed in the washed muscle. If the actomyosin gel is given the form of a muscle fiber, as in Fig. 21, when ATP is added, the structure "contracts" to a small volume within a

minute or two. The actomyosin micells within this actomyosin fiber have a random orientation, and consequently their shortening and mutual approach must lead to isodimensional contraction, that is, to a shortening in all directions. Such a dimensional change, in the language of colloidal chemistry, is simply called "shrinking," "syneresis." If, however, the thread is made more similar to muscle by arranging its micells parallel to the axis (which can easily be done by stretching them, as shown by Gerendás), then, on addition of ATP the thread becomes shorter and wider without losing volume, and similarly to

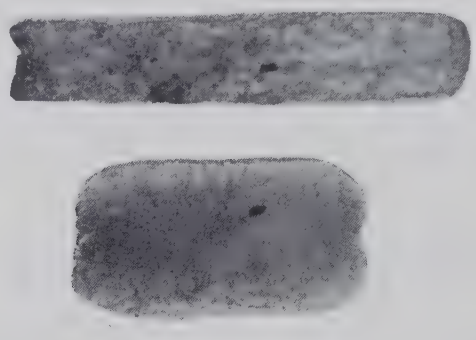


FIG. 22. Same as Fig. 21. Thread was treated according to Buchthal *et al.* and contains micells oriented parallel to the axis (see text).

muscle, it contracts. Figure 22 shows the ATP contraction of such a thread. In this case the micells were arranged parallel to the axis by Matoltsy and Varga, who used the method of Buchthal, Deutsch, Knappeis, and Petersen (1947). This method consists of allowing the actomyosin threads to dry with their ends fixed. The drying thread would have shrunk and shortened if its ends had been free. Being fixed, this could not happen and the result was the same as if it had been allowed to shorten and then had been stretched to its original length again, thereby arranging the micells coaxially.

An actomyosin "solution" is nothing but a very dilute, liquid gel in which the anastomosing filaments form a continuous net. If ATP is added to such a "solution," the slightly opalescent fluid becomes turbid and soon the whole colloid shrinks to a small plug, as shown in Fig. 23(*d*). If the continuity of the net is broken up by shaking the fluid, instead of a "contraction," a violent precipitation is obtained. The slightly opalescent fluid becomes milky and a rough granular precipitate is formed which settles quickly to the bottom, occupying a small volume and leaving a clear fluid behind, which contains neither myosin nor actin [Fig. 23(*c*) and (*e*)]. Salt alone, with ATP, also causes precipitation, but the precipitate formed is very loose and floccular, so here again we must distinguish the weak precipitation, caused by salts

alone, from the violent superprecipitation obtained in the presence of ATP.

The participation of two proteins in the process of contraction is rather remarkable. As we have seen, contraction involves discharge and precipitation. Discharge and precipitation in hydrophobic colloids are mostly caused by ions. In hydrophylic proteins, however, ions have no such action. Precipitation in hydrophylic systems is mostly caused by the interaction of two specially built colloids such as an albumin and its precipitin.

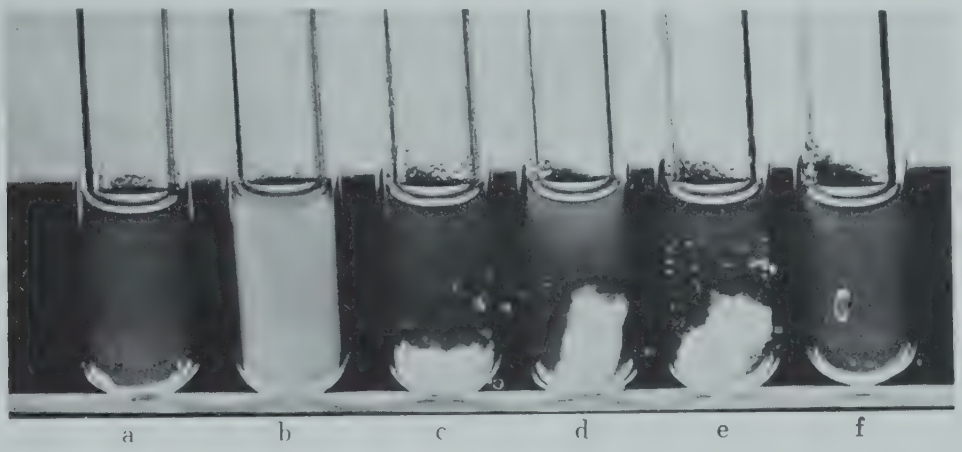


FIG. 23. (*a*) G-actin + myosin; (*b*) myosin + ATP + G-actin; (*c*) myosin + G-actin. ATP added 5 seconds later; (*d*) myosin + ATP + F-actin; (*e*) myosin + F-actin. ATP added 5 seconds later; (*f*) F-actin + myosin. (S. S. Spicer, unpublished.)

Let us look back for an instant and reexamine our trail. We took a strip of living muscle the unique property of which was that it could contract, that is, suddenly change its shape, become shorter and wider, and lift weight or develop tension. Then we took the same muscle, washed it, and then returned its juice to it, and again it contracted, developing the same tension and lifting the same weight as before. Then we showed that what elicited this very specific reaction were ATP and ions. Then we reduced the muscle from a gel to a suspension, and now, instead of contraction, superprecipitation was obtained. Finally we took the contracting structure, decomposed it into two proteins, put those two proteins together again and saw that these combined proteins contract and superprecipitate and even develop not inconsiderable tension (Portzehl and Weber) with the very same highly specific ATP in the presence of the very same ions. This, in the author's mind, leaves little doubt that all these reactions of protein structure elicited by ATP plus ions are essentially identical. It is not mere reiteration if we go over this sequence once more. If we are embarking on the detailed

study of actin, myosin, and their reactions, in the hope of understanding muscle, we must be sure we are not studying a meaningless artificial reaction or system—and serious doubts have been raised in different quarters about this point. Astbury and his associates Perry, Reed, and Spark emphasized the fact that an unoriented actomyosin thread simply shrinks, while muscle becomes shorter and wider in contraction. Supported by EM observation they think that what happens in actomyosin is simply shrinking due to collateral association of the actomyosin filaments, which has little to do with muscular contraction. They also emphasize the slowness of this process compared to the contraction of muscle. Buchthal and his associates were impressed by the observation that a loaded actomyosin thread extends and becomes longer under the influence of ATP while muscle shortens, whether loaded or unloaded. They very cautiously call the reaction of actomyosin threads "volume contraction."

How far an actomyosin thread will imitate the behavior of muscle depends on how far we are able to restore to it the special architecture of the contractile matter, as it is present in muscle. In the A-band of muscle, molecular chains are arranged parallel to the axis and form a very dense, closely packed structure with more than 20 per cent actomyosin. The actomyosin thread mostly contains 97 per cent of water and consists of micells arranged at random with huge lagunae of water between. So contraction of these micells will make the thread shorten equally in all directions while the concomitant discharge of particles will lead to the slow mutual approach of particles, a syneresis. As was mentioned, coaxial arrangement of micells makes dimensional changes similar to muscle. Contraction can also be made very fast if diffusion is excluded as a limiting factor. The ATP, if reaching the thread from one side, will make it bend immediately toward that side owing to the asymmetric contraction at the surface. Single glycerinated psoas fibers contract within a fraction of a second. On the other hand, muscle can easily be made to imitate threads, lose up to 35 per cent of water in contraction,[1] or lengthen under the influence of ATP (Matoltsy and Varga), while Portzehl and Weber found that threads could lift considerable weight, about one-tenth of that lifted by muscle. So the differences in behavior of actomyosin and muscle are no proof of an essential dissimilarity of reactions.

It would be impossible to close this chapter on the chemical disintegration of muscle without mentioning "tropo-myosin," a most interesting new protein, discovered and isolated from muscle in crystals

[1] This is the case when a sartorius is made to persist in the contracted and discharged state, as in contracture produced by freezing and thawing (author, 1949).

by Bailey (1946). The fact that this substance is a member of the creatine-myosin-fibrin group does not prove that it is actually derived from the fibril or has anything to do with contraction. Bailey himself failed to produce evidence to this effect. The quantities of tropo-myosin obtained from muscle are rather small compared to the quantity of myosin. Since this book is concerned primarily with the fibril and its contraction, the discussion of tropo-myosin is outside its scope and will be omitted, however remarkable this substance may be and however important its discovery.

Similarly, the discovery of para-myosin by Hall, Jakus and Schmitt (1945) with all its striking properties and regularities must be omitted until more definite information is forthcoming about its relation to contraction.

Myosin

1. General Properties

Myosin has two unique features: it, in spite of being a hydrophylous colloid, is precipitated by small concentrations of neutral salts, and it has two different enzymic activities. These specific properties are evidently connected with its specific function, and in order to understand this function, contraction, we will have to study them more in detail.

Composition. The elementary analysis (I) of myosin shows the usual figures for protein. An amino acid analysis has been made by Bailey (1944). It shows nothing extraordinary (Bailey, 1950). Its most striking feature is the high content of dicarboxylic acids, aspartic and glutamic, as well as the correspondingly high content of basic amino acids (asparagine, lysine along with 1.7% histidine). Unfortunately, the analysis was made with "myosin," containing unknown quantities of actin. Probably the actin content was not high enough to cast doubt on the correctness of the quoted results.

Ninety-five per cent of the S present in "myosin" was accounted for by Bailey (1937), two-thirds as methionine and one-third as cysteine. Greenstein and Edsall have shown that part of the SH is free, while part of it is masked. The bound SH could be liberated partly by strong urea and almost completely by guanidine. Mirsky found a similar gradual liberation of SH on graduated denaturation by heating.

"Myosin" contains a small amount of lipid matter (Greenstein and Edsall) which is also true of crystalline myosin. Myosin contains a small amount of P, about one P for every 70.00 g (Bailey, 1944; Lajtha, 1948).

Molecular Weight, Shape, and Structure. Both the ultracentrifuge (Snellman and Erdös, 1948a; Weber, 1950) as well as the Tiselius (Erdös and Snellman, 1948) show "crystalline myosin" to be practically homogeneous, monodisperse. Completely homogeneous matter has

been obtained by Guzman Barron (personal communication). The ultracentrifuge, in the hands of Bergold; Bergold and Schramm; Snellman and Erdös, (1948a) Portzehl and Weber showed an S_{20} of 7.1–7.2 and the MW calculation based thereon indicated a MW of 1.5×10^6 g (Bergold; Snellman and Erdös). Measurements of the osmotic pressure by Portzehl and Weber led to a lower value, 840,000 ± 33,000 g. The same authors obtained from S_{20} and D_{20} MW 821,000. Mommaerts (1950) led by angular light diffraction arrived at a similar value, 850,000 g. Osmotic pressure or light scattering involves less incertitude than the ultracentrifuge, and so the value of 840,000 is the more likely to be correct and will tentatively be accepted in subsequent pages. It is rather remarkable that the first MW estimation made by Weber and Stöver two decades ago should have given the correct value MW 10^6 g.

All measurements hitherto published indicate a very thin and long particle with a high axial asymmetry. According to Weber the particles are 22–23 A wide and a hundred times as long; according to Mommaerts (1950) 30 A wide and fifty times as long.

Not much information has so far been obtained from the EM. Most pictures (Rozsa and Staudinger; Snellman and Erdös, 1948b) suggest very thin, long particles beyond the limit of the present resolving power of the instrument. These particles seem to aggregate readily to fibers or needles of different dimensions (Rozsa and Staudinger; Jakus and Hall; Snellman and Erdös). This is shown in Fig. 24, the EM picture of the shaft of a "myosin-crystal," evidently an aggregate of very thin and straight particles. At the side of the crystal the picture also shows smaller and shorter needles or threadlike aggregates. The crystals can be dispersed by ultrasonic radiation (Snellman and Erdös, 1948b) and have fraying ends (Rozsa and Staudinger).

Viscosity and Dispersity. If dissolved in 0.6 *M* KCl at pH 7, up to a concentration of 0.2%, myosin has a normal viscosity (Fig. 24*a*). Above 0.2%, the viscosity begins to be anomalous.

The dispersity of a myosin solution depends on the pH and the nature and concentration of the salt present. Myosin is readily soluble at pH 6.5–7 in strong salt solutions such as 0.6 *M* KCl. If such a solution, containing 2–3% myosin, is diluted with an equal amount of water, a silky sheen appears on mixing, accompanied by an intense double refraction of flow. Both phenomena are due to an association, to a swarm or tactoid formation, and readily disappear on increasing the pH or salt concentration. The swarms must consist of long straight particles with parallel axes. At this stage no particles are visible under the microscope, the distance between the single particles being

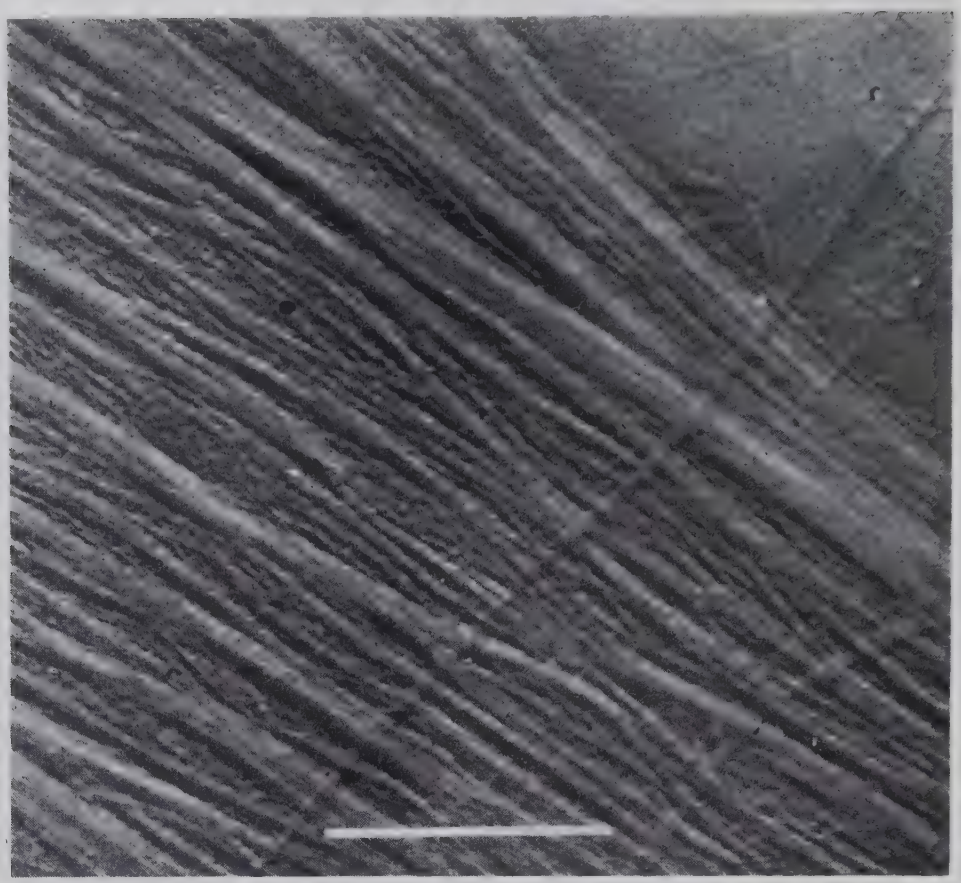


FIG. 24. EMP of the shaft of a myosin "crystal." (Rozsa, Szent-Györgyi, and Wyckoff.)

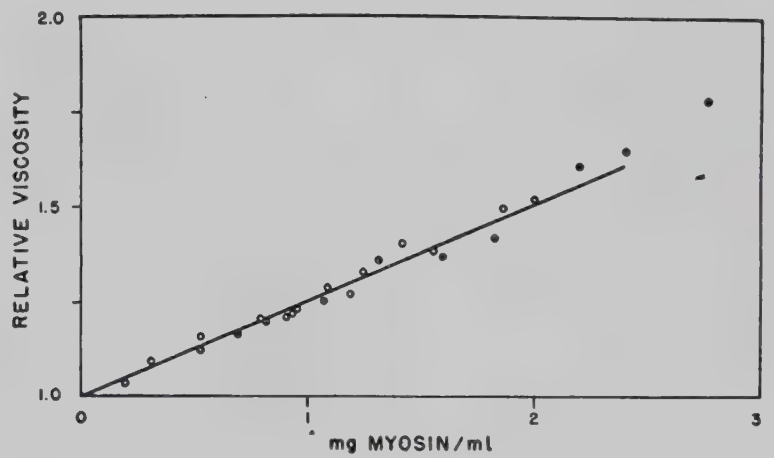


FIG. 24. (*a*) Viscosity of recrystallized myosin in 0.6 *M* KCl at O°C (pH 7). (Guba and Straub, 1943.)

great, the swarms loose. In this state myosin resembles soaps, which in watery solution show a similar sheen and double refraction of flow. On further dilution the swarms become denser and, as the KCl concentration drops to 0.025–0.05 *M*, the particles associate to form microscopic needles, similar to the "crystals" of tobacco mosaic virus. If diluted

further, the crystals begin to swell, intermolecular distances increase again, and below 0.001 *M* KCl the protein dissolves with endless swelling, forming a very viscous solution with a strong double refraction of flow. The EM shows now a continuous net of fibers (Snellman and Erdös) which can be spun out on an ordinary centrifuge (Rozsa, personal communication); 0.025 *M* KCl precipitates it again, while alkali or strong salt brings it into real solution. It also readily spreads on the surface of such strong salt solution (Munch-Petersen).

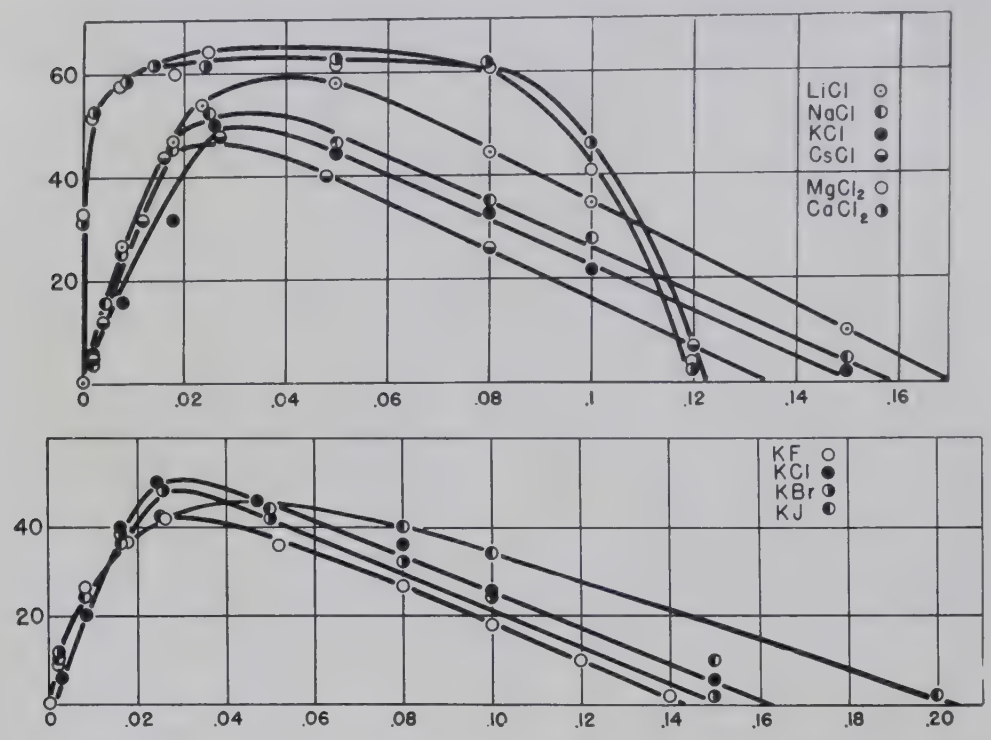
One rather remarkable point about this behavior of myosin is that it indicates that we can "discharge" and precipitate myosin without causing its thin, long, and straight particles to fold up and lose their linear configuration, though such a linear form is the most improbable one for such a very thin and long particle exposed to heat agitation. Evidently the particles are kept straight by their high charge density and hydration, while the equilibrium distance between particles is decided by their over-all charge, which has its origin in the slight excess of dissociated acidic over alkaline groups and is modified by the ions present. It is evidently only this over-all charge which becomes depolarized when the myosin is precipitated by KCl. The charge density remains intact and keeps the particles extended.

Stability. Myosin is stable in one way, but rather unstable in another. Snellman and Gelotte have found it to deteriorate rapidly, the molecules to break up, and preparations to become inhomogeneous in the course of manipulations. The absorption power toward ions suffers quickly on storage, while its ATP-ase activity remains unchanged in 0.6 *M* KCl at pH 7 and 0°C for weeks. If this solution is incubated for an hour at 37°C, its viscosity doubles, indicating denaturation which can be prevented by ATP which, as Engelhardt and Ljubimowa observed, stabilizes myosin. If the salt solution of myosin is diluted with equal parts of glycerol, contractility may be preserved unchanged for months in the deep freezer (—20°C). If the salt solution is kept 0°C overnight, a small quantity of insoluble precipitate is formed which slowly settles. If eliminated it will be replaced by another precipitate by the next day. Weber (1950) observed a fraction in the ultracentrifuge which he attributed to denaturation, and he showed that this denaturation was an all-or-none process, there being no intermediate forms between the denatured and native protein. In strong urea (30%), the myosin particles coil up reversibly into a globule (Snellman and Erdös, 1948a) while prolonged treatment with strong urea makes the particles disintegrate into heterodisperse smaller units (Snellman and Erdös), as observed earlier by Weber and Stöver who found an average MW of 10^5 g.

Myosin is readily destroyed by strong acid or alkali and denatures

quickly even at its isoelectric point (pH 5.4) if salts are present, but it is fairly stable at this pH in the absence of salts (Spicer, 1950). It can be freeze-dried in the native state (Spicer, oral communication). Ammonium sulfate precipitates it at half saturation in the native state.

Complexes. Myosin forms insoluble complexes with glycogen but



FIGS. 25 and 26. Precipitating action of different salts. Abscissa, molarity of salt; ordinate, precipitation in arbitrary units (log of turbidity). (N. K. Sarkar, unpublished.)

not with starch or dextrin. The complex is dissociated by ATP (I). It is not impossible that the formation of this myosin-glycogen complex and its dissociation by ATP play a part in the regulation of carbohydrate metabolism which serves to maintain the ATP concentration in muscle.

X-Rays. Needless to say, any book on muscle is necessarily very incomplete without exhaustive treatment of X-ray data. The author feels incompetent to deal with this aspect and refers readers instead to Astbury's Croonian Lecture and his more recent summary (1950) the first sentence of which reads: "No X-ray diffraction photographs have yet been obtained of muscle in the very act of shortening or relaxing" which may excuse presenting this book as incomplete as it is.

2. Myosin and Ions

General Remarks. The precipitating action of salts is shown in Figs. 25 and 26. Within the same homologue series the action reveals

only minor differences, which show a continuous progression according to the position in the periodic system. K. Laki (1942) has connected these differences with the electronegativity of the atoms concerned. Myosin is unable to distinguish between K and Na (Erdös, 1946).

The colloidal state of other hydrophylous colloids may also be influenced by ions. In fact the author's very first biochemical paper, written under the guidance of L. Michaelis thirty years ago, dealt with the shift of the isoelectric point of casein under the influence of salts. These effects observed in casein or other hydrophylous colloids are very weak compared to those observed in myosin. Nevertheless, they are there (Erdös 1942), and when Nature made myosin she did not apply new principles, but simply built this substance in such a way that certain general properties of the colloids of this class were very strongly brought to the fore.

The Acid Titration Curve. Among ions the H and OH ions have specific actions, and their study asks for specific methods, such as the establishment of the "titration curve." This experiment consists of taking a definite amount of protein, adding known amounts of acid or alkali to it, measuring the pH, and making a plot of the two values, as shown in Fig. 27. If acid were added to pure water, the pH would rise very quickly and the curve would be very flat. The same is true if protein is present but does not react to the H— or OH ions added, there being no acidic or alkaline group in it which would change dissociation at that pH and bind the H^+ or OH^- added. The steepness of the slope indicates a dissociating group and the middle of the slope corresponds to the pK of that group.

Figure 27 is Mihályi's titration curve of crystalline myosin. It is not essentially different from that of Dubuisson, made a decade earlier (1941) with "myosin." The curve with empty circles is salt-free myosin. It shows three steep slopes, one on the left, one on the right, and one in the middle. The first corresponds to the dissociation of the free COOH groups, the second to that of the basic groups (guanidine, arginine, lysine). Lying at unphysiological pH's these will not concern us any further here. What is of possible physiological interest is the steeper slope in the middle at neutral reaction, only faintly indicated in Dubuisson's curve. The middle of the slope corresponds to the pK of imidazole, and so it is evidently the histidine which dissociates here and to which the protein owes its ability to bind H or OH ions at neutral reaction.

The other curves in the same figure show the acid titration curve of myosin in the presence of varied concentrations of KCl. On the whole, salts make the curve flatter. In the acid region, where the ac-

tion of Cl predominates, the shift in the curve is proportional to the log of the salt concentration, indicating that there is no specific interaction between Cl ions and the protein. In the alkaline region where the action of K ions predominates, maximal shift is reached at a relatively low salt concentration, indicating a special affinity between K^+

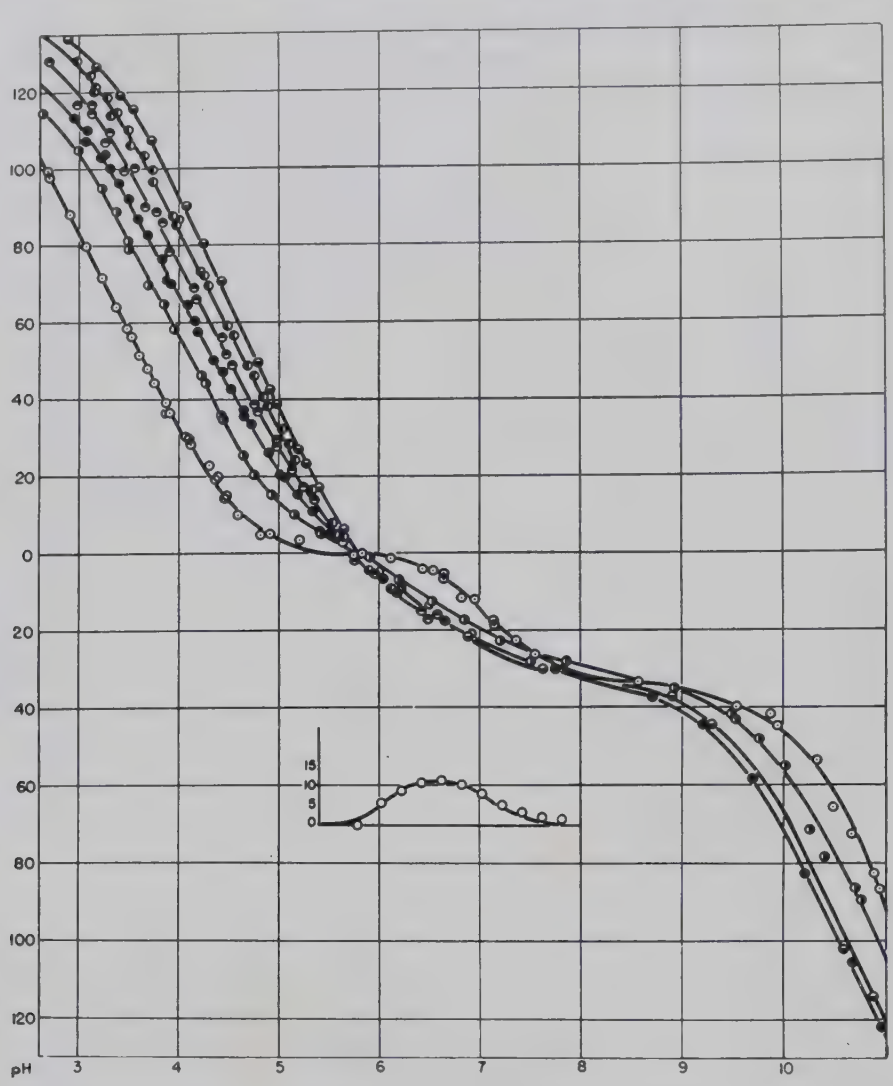


Fig. 27. Acid titration curve of myosin. Ordinate: Acid or base equivalents (or number of protons bound) added to 10^5 g of myosin. Empty circles, salt-free; half-circles, left side white, 0.05 *M* KCl; black circles, 0.15 *M* KCl; circles with lower half white, 0.3 *M* KCl; right half white, 0.6 *M* KCl; upper half white, 0.12 *M* KCl. Inset, difference in titration in the presence and absence of salts. (Mihályi.)

and protein. The same is true of the imidazole in the middle. The difference between the titration curve of salt-free myosin and the curve with maximum salt effect is shown in the inset. It indicates 12 acid equivalents corresponding to the dissociation of 12 imidazoles per 100,000 g myosin, which agrees well with the analysis of Bailey (1944)

according to which there are 11 histidines in 100,000 g myosin. NaCl gave identical curves with KCl.

The total number of dissociated groups at neutral reaction, according to the curve, is very high. About 160 dissociated carboxylic groups are present in the 100,000 g myosin at neutral reaction, slightly exceeding the number of dissociated alkaline groups. If the MW of myosin is 840,000, this means 2500 dissociated groups per molecule, about 1 for every angstrom of length, an exceedingly high charge density which must entail a very strong hydration, keeping the very thin molecules in their otherwise highly improbable linear configuration.

Isoelectric Point (IP). The great number of charges generated by dissociation cannot be expected to have a distant action, since the opposite charges neutralize each other. Repulsion between two particles is decided by the over-all charge. If the over-all charge is 0, the attractive forces are no longer compensated, particles approach, and the colloid precipitates. If the particle, at pH 7, has a negative charge, owing to the prevailing dissociation of the COOH groups, it can be rendered isoelectric by the addition of acid which represses the dissociation of the acidic groups. At pH 5.4 the number of positive and negative groups is equal, the protein is isoionic and isoelectric. At this IP, the myosin shows no electric convection while it moves toward the anode at a higher and toward the cathode at a lower pH. We can also depolarize the over-all charge, leaving this dissociation unchanged by the addition of ions which are adsorbed. If, at neutral reaction, myosin absorbs K ions which decrease its over-all charge to 0, the colloid precipitates in spite of the prevailing dissociation of the COOH which may remain unaltered. The precipitation will give us no information about the number and nature of charges still present, but will merely indicate the minimum of the over-all charge. As shown by Mihályi's curve the ions added also induce a change in the dissociation of the protein, but this change, around the physiological pH, is small.

In order to obtain information about the number of adsorbed ions we must combine both methods, discharge and titration. We may start by measuring the IP of myosin at varied salt concentration, as done by Sarkar. The results of one of his experiments is reproduced in Fig. 28. The curves show the precipitation of myosin at different [KCl], while the plus and minus signs indicate the electrophoretic motion at the corresponding pH, showing that the precipitation observed was in every case isoelectric and was due to the neutralization of the over-all charge of the particles.

As Fig. 28 shows, in the absence of salts, the IP of myosin is at pH 5.4. Here the protein is precipitated, whereas at pH 7, dissolution is al-

most complete; 0.025 *M* KCl shifts the IP to pH 7. Maximal precipitation now occurs at neutral reaction, while the protein has become soluble at its former IP, pH 5.4. This simple observation tells us that when "KCl precipitates myosin" it merely shifts its IP from the acid side to the neutral pH. This means that the myosin has lost some of its negative charges (or picked up positive ones). KCl could induce such a change either by promoting the dissociation of the imidazole

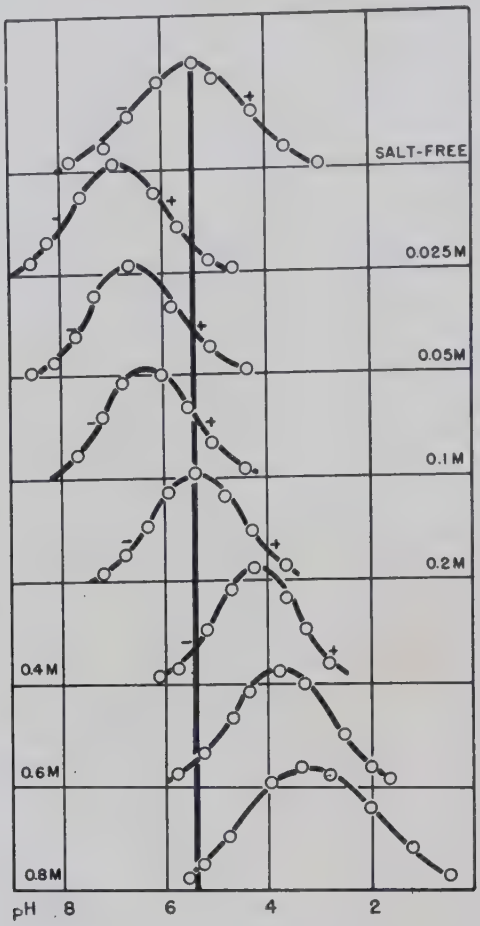


Fig. 28. Isoelectric precipitation of myosin at varied KCl concentrations. Ordinate, precipitation in arbitrary units. Plus and minus signs indicate positive or negative charge as judged by electric convection. These signs relate to the values of the abscissa. (Sarkar.)

groups or else by the "adsorption" of the K^+ to the protein. The acid titration curve shows that KCl depresses the dissociation of imidazole rather than promotes it, and so we must suppose that the protein has bound K^+ to which it has a special affinity, which it binds without the simultaneous adsorption of Cl^- ions.

The doubling of the [KCl], from 0.025 to 0.05 *M* caused no further shift of the IP (Fig. 28), showing that the affinity of the myosin to K^+

was satisfied at the lower [KCl] which was sufficient to provide K^+'s for the complete neutralization of the negative over-all charge of the protein. The protein has bound K^+ with a great affinity in a quantity equivalent to its own negative over-all charge, which was reduced to 0 without considerably changing the dissociation of the protein.

As Fig. 23 shows, not only does an increase of [KCl] cause no further shift on the IP to the left, but it even causes a shift in the

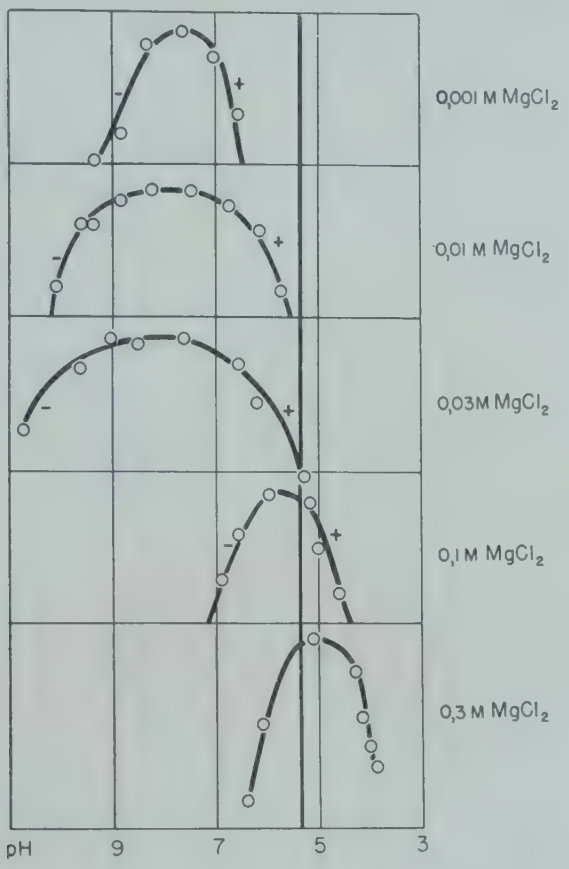


FIG. 29. Isoelectric precipitation of myosin at varied $MgCl_2$ concentration. Same as Fig. 28.

opposite direction, which means that the protein begins to acquire negative charges. Since the acid titration curve reveals no changes in the dissociation of the protein, we must suppose that this shift is now due to the adsorption of Cl^- ions. At the highest [KCl] studied the IP shifted even below its original value making the protein soluble again at neutral reaction. This shift of the IP with increasing [KCl] is summed up in Fig. 30 (half-circles), which shows that the IP tends to reach pH 3 asymptotically.

It is possible to calculate, approximately, the number of these adsorbed ions.

According to the titration curve 100,000 g of myosin, in the presence of small amounts of KCl, has 24 negative charges at pH 7, as many acid equivalents being needed to bring the pH from 7 to 5.4. If at pH 7 the myosin had 24 negative charges and 0.025 *M* KCl made it isoelectric, then 24 K^+ must have been bound to 100,000 g of it (and 16 to 70,000 and 4 to 18,000), without the simultaneous adsorption of Cl^-.

Muscle contains also Ca and Mg. Ca interests us little here because its quantity is small and its affinity to actin great, thus most of it must be bound to actin. Mg has less affinity to actin, is present in the relatively high concentration of 0.11–0.12 *M* (Dubuisson, 1942; Steinbach) and has a high affinity to myosin. If myosin binds 24 equivalents per 100,000 g, most of the Mg present must be bound to it. The influence of Mg and Ca on the charge of myosin has been studied by Snellman and Erdös (1948c) who found the protein charged positively but were unable to find any isoelectric precipitation. Figure 29 shows the results of Sarkar. As can be seen, even 0.001 *M* $MgCl_2$ has a very strong effect, shifting the IP to pH 8; 0.0005 *M* would have done the same, while increasing the concentration to 0.01 or 0.03 *M* does not make the shift any greater—it only widens the zone of precipitation. Here again, at the higher $MgCl_2$ concentration, the Cl^- makes itself felt and shifts the IP to the right, as shown in Fig. 30 also (empty circles).

The strong shift to pH 8 by the small $MgCl_2$ concentration indicates a great affinity between Mg and myosin. As Fig. 30 shows, on increasing the salt concentration, the IP shifts back to the lower pH with a steeper gradient than was the case with KCl. We have to conclude that not only has Mg a great affinity to myosin but that the bound Mg also increases the affinity of myosin to Cl^-. The Cl is bound strongly not only until it has neutralized the positive charge induced by Mg, but even beyond. This conclusion is very clearly borne out by the third curve in Fig. 30 (full circles). In this experiment 0.001 *M* $MgCl_2$ was added to all samples, and the KCl concentration was varied. The curve, that is, the shift of the IP to the lower pH, is exceedingly steep, showing that the Mg has very greatly increased the affinity of the myosin to Cl^-, which is now bound much more strongly than in the sole presence of K as cation. The Mg must have entered into intimate relations with the myosin, and the Mg myosinate thus formed is a new substance with an excessive affinity to Cl^-.

These observations bring out two important points. First, they tell us that we cannot treat ions simply as charged points and that their effect is not additive. Every ion induces new and specific changes in

the protein which modify its affinities toward other ions. Secondly, the effect of anions is as important as that of the cations with which they are always added. If we talk about "balance" of ions, anions have to be taken into account, and if we are dealing with the balancing effect of monovalent cations toward divalent cations, evidently the anions are in the picture too and we are dealing less with cations balancing one another but with their balancing anions in a different way. Our observations also make it probable that the relatively weak affinity of myosin to Cl in the sole presence of KCl was not the affinity of myosin

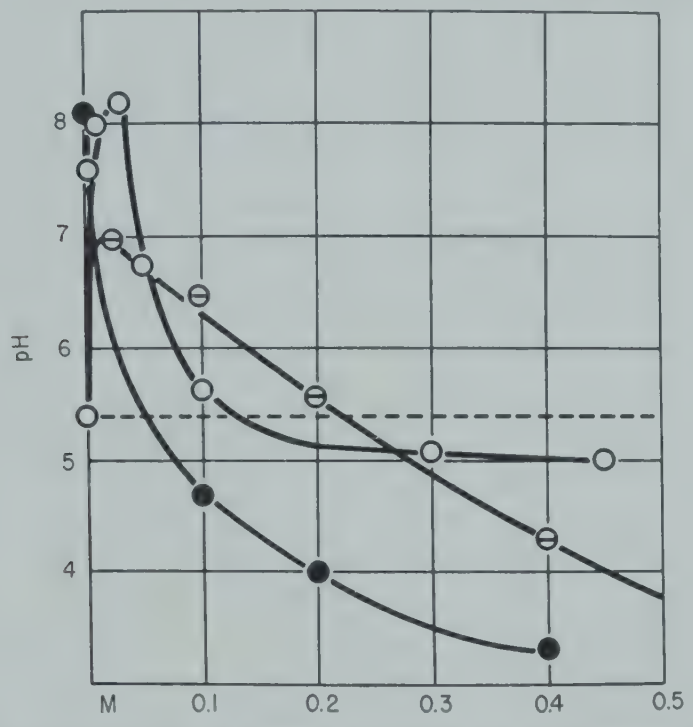


FIG. 30. Isoelectric point of myosin at varied salt concentrations. Half-circles, KCl; empty circles, $MgCl_2$; full circles, varied KCl plus 0.001 *M* $MgCl_2$. (Sarkar.)

but that of the K myosinate. ATP, which is a polyvalent anion, behaves similarly to Cl: it is not bound by myosin at all in the absence of salts, poorly bound in the presence of KCl (the quantity of ATP adsorbed depending on the quantity of adsorbed K), more strongly bound in the presence of $MgCl_2$, and the most strongly if both K and Mg are present (Hermann).

Chemical Methods. We can obtain some information about the binding of ions also by direct chemical analysis. Banga (I) proceeded as follows. She precipitated myosin at pH 7 with KCl, separated the precipitate in the centrifuge, washed it with alcohol, and analyzed it for K and Cl. Since the supernatant fluid was rejected, in principle the method was similar to compensation dialysis. Banga found that

the precipitate contained 3–5K for every 18,000 g of myosin and no Cl, if the precipitation was made with 0.025 *M* KCl, a value in good accord with Sarkar's results. She repeated this experiment at varied KCl concentrations. Her results are summed up in Fig. 31. If log [KCl] was plotted against adsorbed K, a straight line was obtained which showed a break at the point where the myosin was maximally precipitated and had no over-all charge. Up to this point no Cl was adsorbed, but from this point on the Cl was adsorbed in increasing quantities. The flatness of the curve below the breaking point suggests a strong binding of K. The lower curve in Fig. 31 was taken with a myosin stored for 48 hours and shows that adsorption is a subtle quality of myosin and is easily lost on storage.

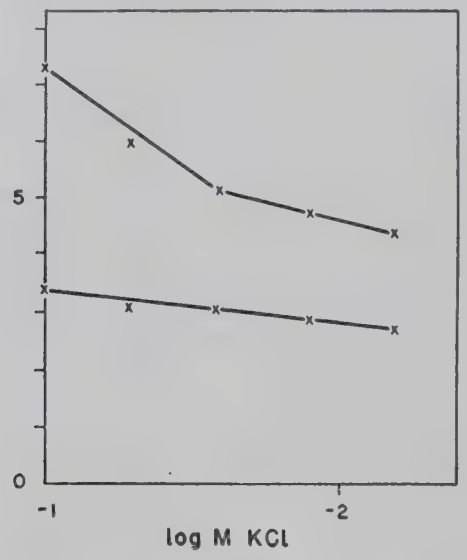


FIG. 31. The adsorption of K to myosin. Ordinate, equivalents of K bound by 18,000 g of myosin at pH 7; upper curve, fresh myosin, 24 hours after the death of the animal; lower curve, the same myosin 48 hours later. (Banga, I.)

Myosin and ATP. ATP is a tetravalent anion. Out of its four acidic groups approximately three are dissociated at neutral reaction. Various methods have been used by different authors to study its binding by myosin. None of them is free of reproach but, taken together, they give some idea of the relation of this protein and the nucleotide.

Banga and Hermann used the direct chemical method, precipitating myosin with alcohol in the presence of ATP and analyzing the precipitate. Banga showed that the presence of actin does not alter the affinity of myosin to ATP. Hermann showed that ATP adsorption is a subtle quality of myosin, rapidly lost on storage. She also showed that in the entire absence of salts, myosin does not bind ATP at all. In the presence of K there is moderate binding which is proportional to the

quantity of K bound by the protein. Mg very greatly enhanced this adsorption. Its action consisted less of increasing the quantity of bound ATP than of decreasing the ATP concentration necessary to cause saturation. Adsorption was the strongest if both Mg and K were present. Eighteen thousand grams of myosin was found to adsorb 1–3 moles of ATP, the lower values being the most probable ones.

Sarkar found that the IP of myosin, shifted to pH 7 or 9 by small concentrations of KCl or $MgCl_2$, was readily shifted back to its original value (5.4) by very small concentrations (0.003%) of ATP, showing that this nucleotide was bound in quantities equivalent to the metal adsorbed. If ATP was added at higher KCl concentrations in which the adsorption of Cl prevailed, shifting the IP to 5.4 or below, ATP

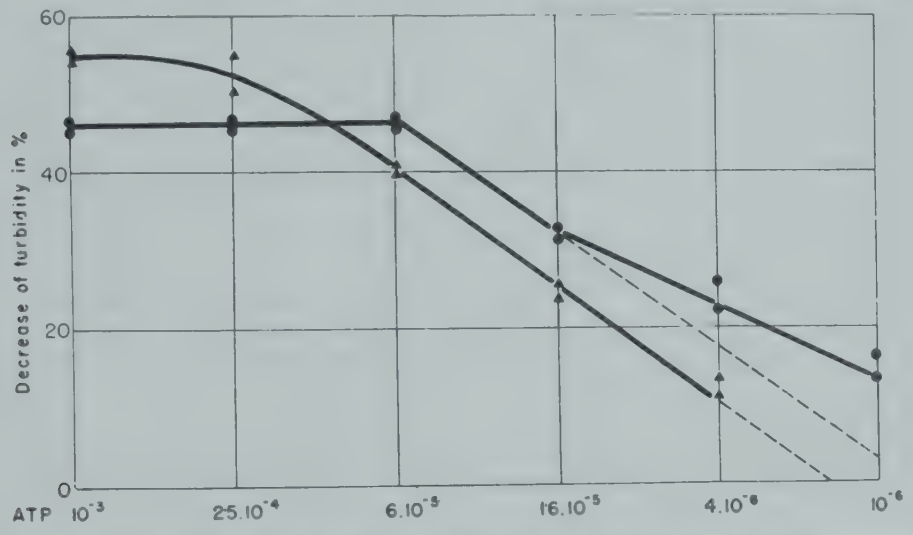


Fig. 32. Turbidity of myosin precipitated by KCl at varied ATP concentrations. (M. Borbiro, unpublished.)

caused no further shift, indicating that, if bound at all, it replaced adsorbed Cl ions at its fixation.

Borbiro (unpublished) "precipitated" myosin at pH 7 by adding 0.05 *M* KCl. ATP "inhibits" this precipitation by shifting the IP back from pH 7 to 5.4. Borbiro measured this "inhibition" by measuring the turbidity in the presence of varied ATP concentrations. Her results are summed up in Fig. 32 (triangles). As can be seen, the myosin gradually reached saturation at higher ATP concentrations. If the experiment was repeated in the presence of 0.001 *M* Mg (circles), the same effect was produced by smaller ATP concentrations, half as much ATP being needed for the same effect as before. Saturation was reached with a sharp break indicating a high affinity of the Mg myosinate to ATP. At this point the solution contained 1 mole of ATP per

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18,000 g myosin. The ATP ceased to have an effect if there was less than 1 mole per mole (10^6 g) of myosin. Muscle contains 1 mole of ATP for every 18,000 g of myosin, which ATP can thus be expected to be bound mostly to myosin.[1]

By taking everything together, there can be little doubt that ATP forms a complex with myosin. The link must be rather intimate, for Ravikovich finds a shift in ultraviolet spectrum of myosin toward the shorter wavelength under the influence of ATP. Mommaerts attempted to calculate the bond energy and found it considerable (10,000 cal).

The nature of the bond between myosin and ATP is unknown. Binkley assumes an esterlike bond, but Fabry-Hamoir could find no disappearance of acidic groups. Bailey and Perry assume a link between SH groups and ATP, but produce no evidence in favor of this. Ács, Biro, and Straub showed that myosin also binds other inorganic polyphosphates, the adsorption being promoted by Mg. So, evidently the phosphate end of the ATP molecule is involved in the complex formation which seems to be mediated by metal ions linked to the protein.[2]

Conditions in Muscle. These must be rather complex. At any rate, the usual description "proteins have a negative charge owing to the prevailing dissociation of COOH groups" does not correspond to what we may expect. The prevailing dissociation of COOH groups does lend a negative charge to myosin, but this will be compensated by the Mg ions adsorbed. The fixed Mg ions, in turn, will cause the adsorption of ATP, which restores the negative charge to the particle. Muscle contains enough ATP to provide every 70,000 g of myosin with

[1] Ács, Biro, and Straub attempted to estimate the dissociation constant of the ATP-myosin complex by drawing conclusions from the dissociation of actomyosin, induced by ATP. They calculated that 1 mole of ATP had to be bound to 140,000 g of myosin to cause dissociation. This, compared to the total quantity of ATP needed to dissociate the actomyosin, led to a low dissociation constant for the ATP-myosin complex. Ács, Biro, and Straub concluded that 9 out of 10 ATP molecules have to be free in muscle, unbound to myosin. The constant was somewhat decreased by Mg but not by Ca. These results are at variance with those of Mommaerts (1950*) who found that 1 mole ATP present in the solution of 300,000 g actomyosin suffices to cause complete dissociation. Moreover, the experiments of Ács, Biro, and Straub were made in the presence of high KCl concentrations which lend a strong negative charge to myosin and allow no conclusion to be drawn on conditions in muscle. Furthermore, no attention was paid to the freshness of the myosin preparation.

[2] Mihályi and the author also found that myosin strongly binds Calgon, (polymetaphosphate). Calgon makes myosin precipitate more strongly under the influence of ions, but does not interfere with the binding of ATP, and its own binding is not dependent on Mg and seems thus to have a different mechanism from the fixation of ATP.

4 moles, one ATP for every 18,000 g. The negative charge of the protein thus will be due directly to the adsorbed ATP, and any decrease in the ATP concentration will cause a decrease of this charge, favor association of the myosin with actin, and therefore favor contracture. All this makes a rather complex pattern of charges, leaving the protein particle with a negative over-all charge which is finally balanced by the positive ions, chiefly K, located in the water surrounding the particle. This ionic atmosphere surrounding the particle and the adsorbed ions are in equilibrium; any change in the ionic balance of the medium will cause a corresponding change in the charge of the protein particles and alter their equilibrium distances.

3. Enzymic Activity

Myosin has two enzymic activities, both connected with its biological function: it acts as ATP-ase which splits off the terminal phosphate of ATP (Engelhardt and Ljubimowa) and acts as desamidase (Banga and Josepovits) which splits off the 6 amino groups of adenosine (Hermann and Josepovits). These enzymic functions are completed in muscle by the action of three soluble enzymes present: Kalckar's (1947) acid- and cocktostable *myokinase* which dismutates ADP into ATP and AMP,[3] and the two enzymes concerned with the transfer of phosphate from CP to AMP or ADP. It was Lohman (1934) who described this P transfer and it was Banga (1943) who showed that two "phosphopherases" were responsible for it: one mediating the reaction between CP and AMP and the other between CP and ADP.

These enzymic activities allow us to build tentatively a picture of what may happen in muscle: ATP interacts with myosin, causes "contraction," and is dephosphorylated to ADP. The ADP is rephosphorylated by taking on phosphate from CP (in the presence of ATP-CP transphosphorylase),[4] the net result being the splitting of one CP. If there is no CP available, the muscle tries to make the best of the situation and makes one ATP out of two ADP's, transferring the terminal phosphate from one ADP molecule by means of the myokinase to the

[3] Cocktostable enzymes in muscle were first described by Curtis and Ohlmajer in Meyerhof's laboratory. They seem to form a special group of catalysts. Whether the phosphopherases belong to the group or not is still undecided. No MW estimations have yet been attempted to the author's knowledge.

[4] The equilibrium constant for a bimolecular reaction was measured by Banga and found to lie far on the side of ATP, which shows that the function of the enzyme is to transfer P from CP to ADP and not the reverse. The equilibrium constant for the AMP-CP phosphopherase was not measured. If this were found to lie on the side of AMP, it would indicate that the function of the enzyme is to transfer P from creatine to AMP. If it lay on the side of CP, then the two phosphopherases could act as an ADP dismutase, in the presence of creatine.

other, one ADP being dephosphorylated to AMP, the other rephosphorylated to ATP. The AMP may still be saved and rephosphorylated if CP becomes available. If there is none, there is no use for it, and it is discarded by desamination. The resulting inosinic acid has little affinity to the protein and is released while the bond energy of the amino group pays for the bond energy between the nucleotide and the protein.

This accords well with Lajtha's recent observation that creatine phosphate greatly promotes the contraction of actomyosin caused by small concentrations of ATP, provided a soluble enzyme (phosphopherase) is present.[5]

ATP-ase Activity. Many biochemists have found it difficult to believe that a contractile protein like myosin could be an enzyme, and many attempts have been made to separate the ATP-ase from myosin. That the ATP-ase activity might be due to an enzyme adsorbed to myosin was supported by Kalckar's (1944) observation that potato ATP-ase, once adsorbed to myosin, cannot be detached again. Polis and Meyerhof fractionated myosin into a more active and less active component and Kielley and Meyerhof have shown that muscle contains an ATP-ase which is different from myosin. Singher and Meister, by dialyzing at pH 6, prepared a myosin which acted poorly as ATP-ase, though it still looked like myosin. Gergely (1950) found that trypsin rapidly hydrolyzed myosin into two proteins, one of which was precipitated on dilution but had no enzymic activity. The other was not precipitated, but had the full activity of the original myosin. The hydrolyzed myosin had a low viscosity, indicating that both fractions were more or less globular. Cori and Price also reported the separation of myosin and ATP-ase, but the statement was later retracted (Cori). Banga, Guba and the author also reported the isolation of acid-stable "protins" which they made responsible for the enzymic activities of myosin. Later work of Hermann and Josepovits brought no evidence for "protins," casting serious doubts on their existence.[6] Ziff and Moore were unable to separate ATP-ase from myosin by means of electrophoresis. All these failures to demonstrate that ATP-ase and myosin are two different substances tend to prove that myosin itself is the ATP-ase.[7]

[5] It is not easy to separate myosin completely from myokinase and the phosphopherases; their presence is one of the pitfalls of this field, the dangers of which are increased by the difficulties of preparing pure and well-defined ATP.

[6] Guba and the author described a catalyst, "protin 1" which enabled ADP to elicit contraction in actomyosin. The protin 1 had a colored prosthetic group, "fluochrome." The existence of such a dye or catalyst could not be corroborated later by Mihályi (unpublished).

[7] The fact that muscle contains an ATP-ase which is not myosin (Kielley and

If myosin does not fit into the classical picture of enzymes in many ways, this is because the picture was built upon experience obtained with soluble enzymes such as pepsin or trypsine which have the job of performing a certain catalytic function (such as the disintegration of protein) as quickly and completely as possible. Myosin is the first structural protein ever isolated which makes part of a solid structure, of an involved mechanism. Its ATP-ase activity is a function which is called upon only at a definite phase of the activity of the system, and it is equally important that this function should be exerted promptly at that instant as it is that it should *not* be exerted at others. We can even see how this is achieved. Myosin in resting muscle is free, is not linked to actin, and is present as Mg myosinate. Mg myosinate, at the pH of muscle (6.5–7) is not an ATP-ase at all (Banga, 1943; Greville and Lehmann, 1943), the pH optimum of this activity lying far in the alkaline region (pH 9). As will be shown later, excitation brings about a union between myosin and actin. The resulting actomyosin Mg is most active as ATP-ase, actin having shifted the pH optimum from the alkali side to the actual pH of muscle (Banga, 1941–42; Kielley and Meyerhof; Biro and A. G. Szent-Györgyi; Sarkar, A. G. Szent-Györgyi, and Varga). Actomyosin Mg at this pH is even more active than actomyosin K. Actin thus turns Mg from an inhibitor into a promoter of enzymic activity. Myosin as ATP-ase thus becomes activated by "excitation," which makes actin and myosin unite to actomyosin.[8] Little can be gained by showing that Mg myosinate, *in vitro*, free of actin, works too slowly to account for dephosphorylation in muscular contraction (Mommaerts and Seraidarian). The myosin molecule is part of a complex mechanism and its enzymic action is but one of the series of events taking place in a certain order within this mechanism. The fact that free myosin also splits ATP does not invalidate this argument. The fact that the blade taken out of a razor can still cut a hair in two tells me little about shaving. In the intact cell, reactions involved in energy transfer must of necessity be coupled to prevent leakage. If the mechanism is damaged, the

Meyerhof) does not prove that myosin is not ATP-ase. Muscle is not merely myosin, it is a cell, and if liver cells contain ATP-ase which is not myosin, there is no reason why muscle should not. The same remark may apply to Gilmour's observation that the major half of ATP-ase in insect muscle is water soluble and not linked to myosin. The issue could be decided by producing myosin which is contractile but entirely devoid of ATP-ase activity. The existence of such has not yet been demonstrated beyond doubt.

[8] It is even possible that actomyosin Mg acts as ATP-ase only in the contracted state. Banga (1941–42) and the author (I) as well as Biro and Szent-Györgyi accumulated evidence which shows that ATP-ase activity and contraction occur together.

coupling destroyed, and the enzymes turned loose, these will start working without rhyme or reason, dissipating all the valuable free energy as heat. Myosin does not stand alone in this respect. For instance, within a cell the electron detached from succinic acid loses its high energy stepwise, the drop of energy being coupled with the establishment of a high-energy phosphate link. If the system is slightly damaged, as by "washing," a "succinoxidase" is produced which oxidizes succinic acid at an immense rate, senselessly liberating its energy as heat.

It is necessary to bear these physiological and structural relations in mind to avoid unwarranted conclusions from *in vitro* experiments on conditions *in vivo*, while on the other hand these functional considerations may give meaning to apparently senseless reactions, such as the reversion of Mg inhibition by actin.

The ATP-ase activity of myosin depends on its charge, which in turn depends on ions. In the absence of salts, myosin is inactive as ATP-ase, as it is inactive also if there is too much of them. If, for instance, we study the dependence of the ATP-ase activity on [KCl] or [NaCl], we find the optimum at pH 7 around 0.16 *M*, which is isotonic with blood or muscle (Banga and the author, 1943). Ca strongly promotes, and Mg strongly inhibits this ATP-ase activity of myosin, while both Ca and Mg promote the same enzymic activity in actomyosin. Combined, Mg and Ca inhibit (Banga and the author, 1943; Braverman and Morgulis, 1948; Mommaerts, 1947). All ions which do not denature myosin may activate salt-free actomyosin in the proper concentration. Braverman and Morgulis attribute a stabilizing effect to the combined Ca and Mg on ATP-ase activity. They overlook the fact that the concentration of free Ca in muscle can be but very low.

Many students of the ATP-ase activity of myosin must have asked themselves whether the ATP-ase function is not merely the over-all result of transphosphorylation between myosin and ATP, the terminal phosphate of ATP being transferred to myosin and then detached from it later. The author with Banga and with Lajtha tried hard to demonstrate such a transphosphorylation between myosin and ATP, but unsuccessfully. Buchthal, Deutsch, Knappeis, and Munch-Petersen (1949) have shown that if ATP is allowed to act on myosin, a very labile phosphate appears. The quantities of this phosphate were rather small, and a great deal of loosely bound nucleotide was found which makes the results less convincing. Transphosphorylation of myosin was reported also by Szörényi and Chepinoga (quoted from Lipmann).

It is probable that the one P present on about every 70,000 g of myosin is part of a nucleotide strongly bound to myosin (Lajtha, unpublished) and this might be a point to which P could be transferred. A transphosphorylation, even if definitely established, would not answer but merely shift the central problem: how contraction is produced by ATP and how the energy of its terminal phosphate bond pays for it. We will return to this problem later.

AMP Desamidase Activity. It has been noted by Summerson and Meister as well as Ferdman and Nechiporenko that "myosin" desamidates AMP. Banga and Josepovits complicated the situation by connecting desamidation with dephosphorylation and isomerization of ADP. Hermann and Josepovits resimplified the situation by showing that it was the AMP only which was desamidated and it was myosin itself which was the desamidase, or at least this enzymic activity could not be separated from myosin any more than the ATP-ase activity. They also showed that the desamidase activity had a different pH optimum (pH 5.9–6, 1) than the ATP-ase function of myosin and was activated both by Ca and Mg or their combination, while the ATP-ase activity is inhibited by Mg or the combination of Ca and Mg. Evidently these are two different functions of myosin. Hermann's studies make it probable that Banga's results were to be ascribed to complications introduced by the presence of myokinase, a criticism also made by Bailey (1949). Whether Schmidt's desamidase is actually myosin, as Hermann believes, will have to be decided by more extensive experience.

The ATP-ase activity of myosin is linked to the presence of free SH, as shown by Barron and Singer, and Ziff. Myosin, as ATP-ase, can thus be classed with the SH enzymes. Whether the SH group is actually involved in the splitting of the P—O—P link or is merely needed for the good health of the molecule is undecided. It is also unknown so far whether the presence of free SH is also a prerequisite of the desamidase activity.

Menne described a third enzymic activity of "myosin," dependent on the presence of a coenzyme, responsible for the formation of creatine out of arginine and histidine. No efforts have so far been made to separate this activity from "myosin."

Chapter VII

Actin

1. General Properties

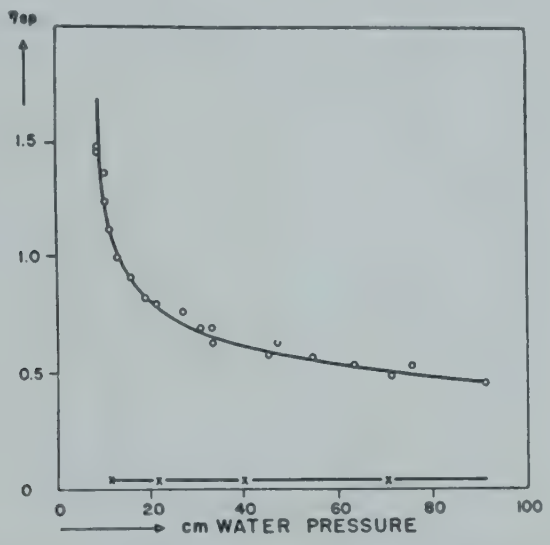
The contractile matter consists of three-fourths myosin and one-fourth actin. One gram of muscle contains 25–30 mg of actin which is 12–15 per cent of the total protein (Balenovic and Straub). Like myosin, actin also has a unique property which is evidently connected with its biological function: it can exist both in globular and fibrous form which can be transformed into each other, reversibly, at a high rate, at a neutral reaction, and at the temperature of the body. Insulin is the only protein which shares this property of having a globular and fibrous state, the transformation taking place at high temperature and acidity (Waugh). We owe most of our knowledge of the properties and reactions of actin to the studies of Straub (1941–43) and his associates whose papers will be quoted freely.

Actin, as extracted from muscle, is a typical globular protein.[1] It is easily soluble in water, giving a limpid solution of low and normal viscosity [Fig. 33(*a*)]. Elementary analysis yields values of C, H, N, and O typical for protein, and no P. Its IP is at pH 4.7 where it precipitates. At its IP, actin is fairly stable in the absence of salts, but readily denatures in their presence. It is destroyed even by weak alkali, denatures at 50°C, and is most stable around neutral reaction. Its amino acid analysis was attempted by Feuer, Molnár, Pettko, and Straub, but until criteria of purity are more definitely established, such data have but limited value. The high dicarboxylic and basic amino acid content is rather striking.

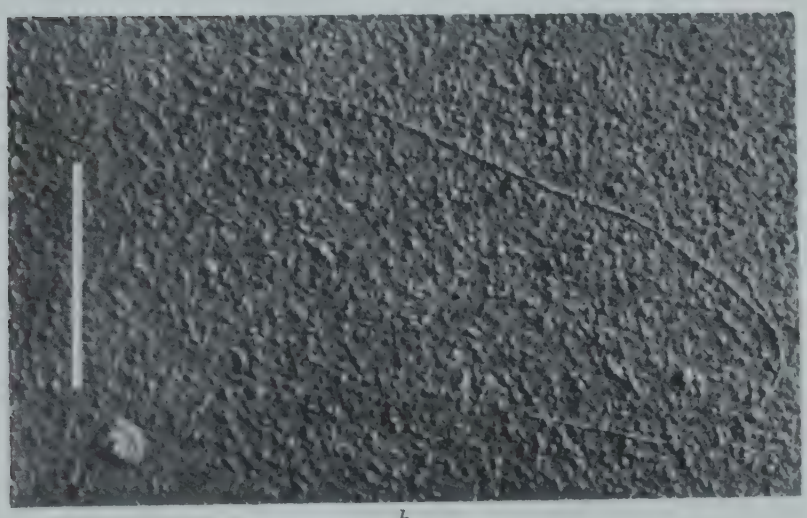
The tryptophan content of actin indicates a minimum MW of

[1] The author repeatedly observed the appearance of crystalline platelets in isoelectrically precipitated solutions of G-actin while the protein denatured and precipitated. The crystalizing substance seemed to belong to the group of cerebrosides. If this finding can be verified, it will mean that actin, as we know it, is actually a lypoprotein consisting of protein and a cerebroside.

70,000 g. Since the protein readily passes through a 10% collodium membrane which retains hemoglobin and partially lets through egg albumin, the MW is within the range of 70,000 g, a rather common MW for simpler proteins. The $S_{20} = 4$, found by Weber (1950*) agrees with this assumption.



a



b

FIG. 33. (*a*) Viscosity of globular and fibrous actin at various pressures. Crosses. 2 mg globular actin per ml, in 0.2 *M* KCl. Circles, 3 mg of fibrous actin per ml in distilled water at O°C. (Straub. 1943.) (*b*) EMP of globular actin in the process of polymerization. Note growing F-actin filament. (Rozsa, Szent-Györgyi, and Wyckoff, unpublished.)

Colloidal criteria for the shape of molecules are rather crude. The author recalls a conversation with H. Staudinger, in which this pioneer of macromolecular chemistry took out his pencil, saying "here is a spherical particle." The joke was that this was no joke. The pencil's

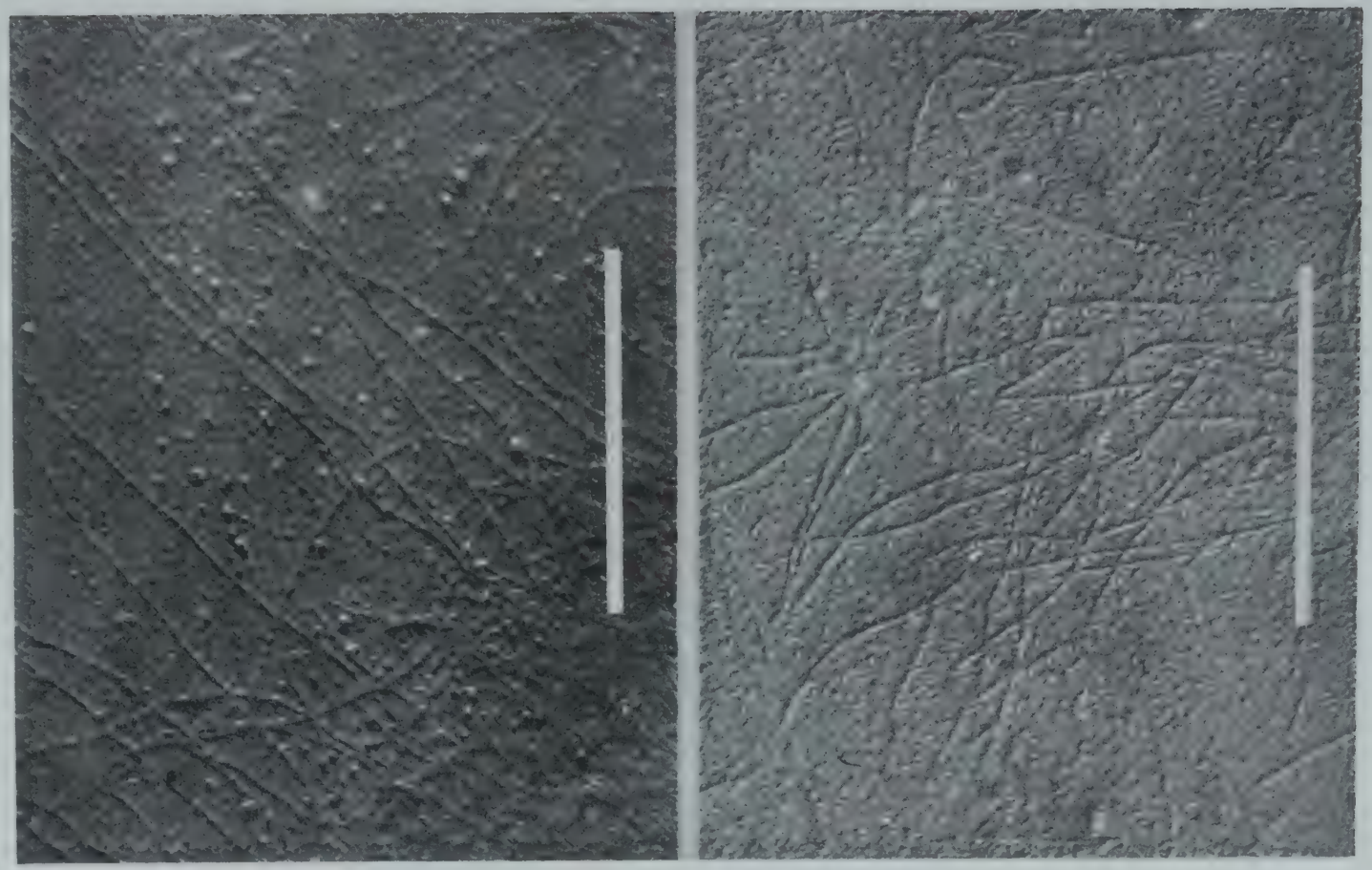
axial asymmetry of 1:20 would still allow the classing of colloidal particles of this shape among spherical colloids. Actin, probably, is really spherical, or at least not very far from it. The single particles are too small to be seen in the EM. If, however, an actin solution is dried down, it collects in little aggregates of different size. The smallest ones still visible can only be composed of a few molecules. All these aggregated are round (Rozsa and Staudinger) as also shown in Fig. 33(*b*) where one fiber is seen lying in the middle of a sea of dried globular actin. If the single particles were asymmetrical, the smallest aggregates would be elongated, which is not the case. The same is shown in Fig. 36.

I have enlarged upon this question because it is important for the understanding of the physical role of this protein. A round shape means mobility. Physical properties of G-actin indicate that the actin

Fig. 34. EMP of F-actin. (Rozsa, Szent-Györgyi, and Wyckoff, 1949.)

globule is not only round but actually is a rather solid, hard little object, fit to act as a ball-bearing. Contrary to other structural proteins, actin, in this G form and at pH 7, is not precipitated or denatured at room temperature by the addition of alcohol or acetone, but precipitates in the native state if the acetone solution is acidified. The water solution can also be dried to a thin sheet in the desiccator without damage and will readily dissolve again. All this indicates that the particle is compact and does not contain water-filled spaces. If any colloid deserves the affix "globular," actin, in this form, certainly does and thus will be called "G-actin" ("G" for globular) to distinguish it from "F-actin," the fibrous form ("F" for fibrous).

If a small concentration of a neutral salt, (e.g., KCl), is added to the watery solution of actin, a striking change takes place within a few minutes: the protein is transformed into a typical fibrous colloid



a *b*

FIG. 35. (*a*) EMP of fibers of F-actin, formed out of G-actin. (*b*) Filaments of blendored muscle. (Rozsa, Szent-Györgyi, and Wyckoff, 1949.)

[Fig. 33(*a*)]. It now has all the characteristics of this group: a beautiful double refraction of flow, which persists for some time after shaking; a strong Tyndall effect; a fairly high and anomalous viscosity; and thixotropy. More concentrated solutions set to a gel and liquefy on shaking. In this form actin readily sediments in the high-speed centrifuge and is easily retained by relatively coarse filters, such as a paper filter coated with a thin sheet of colloidal caolin, which lets G-actin through without resistance.

The EM, as shown by Jakus and Hall, reveals the presence of long, practically endless, smooth filaments of 80–140 A diameter (Fig. 34). Some of the filaments show a striking resemblance to the filaments of the muscle fibril obtained by physical disintegration after the dissolution of the myosin [compare Fig. 35(*a*) and (*b*)], which makes it seem probable that these muscle filaments are nothing but actin threads.

2. The G-F Transformation

Biological structures are built of "fibrous" proteins. It is thus of primary interest to understand what a "fiber" is, and actin with its G-F transformations seems to offer a splendid material for this study.[2] So far we have seen the two ends of the process. We have seen the small globules into which actin was decomposed during its extraction and have seen the ready-made fibers built of these globules, apparently homogeneous (Fig. 35), giving no information about the details of their architecture or the mechanism of their formation. The fact that these fibers are built of corpuscles is of primary importance, but what one would like to know is the blueprint of this construction—how the globules are fitted together to make a fiber. How are they fitted together and by what sort of forces are they held together to develop the tensile strength which is required in muscle? One could hope to find an answer to these questions by catching the fibers, so to speak, red handed, in the process of formation. The problem is how to make such a preparation of polymerizing actin without any agitation which could destroy those very subtly formed structures when the single molecules are being fitted together. This problem was solved by Rozsa, Wyckoff, and the author (1949) by a simple trick which con-

[2] It is important to distinguish two classes of fibrous proteins. One sort, such as actin, is elaborated by the cell to build its structure. The other sort, such as silk fibroin, is secreted by a special organ and is destined to perform the static mechanical functions of a thread of great tensile strength outside the body, to build the cocoon or let a spider descend. The latter group gives us no information as to the nature of the first group.

sisted of adding a mere trace of salt to the diluted solution of G-actin, far from sufficient to cause polymerization. If this solution was spread on glass or collodium and allowed to dry, the salt and actin concentration reached the critical limit and started up fiber formation just before the last trace of water evaporated. At the next instant, however, the last trace of water was gone and the whole structure was

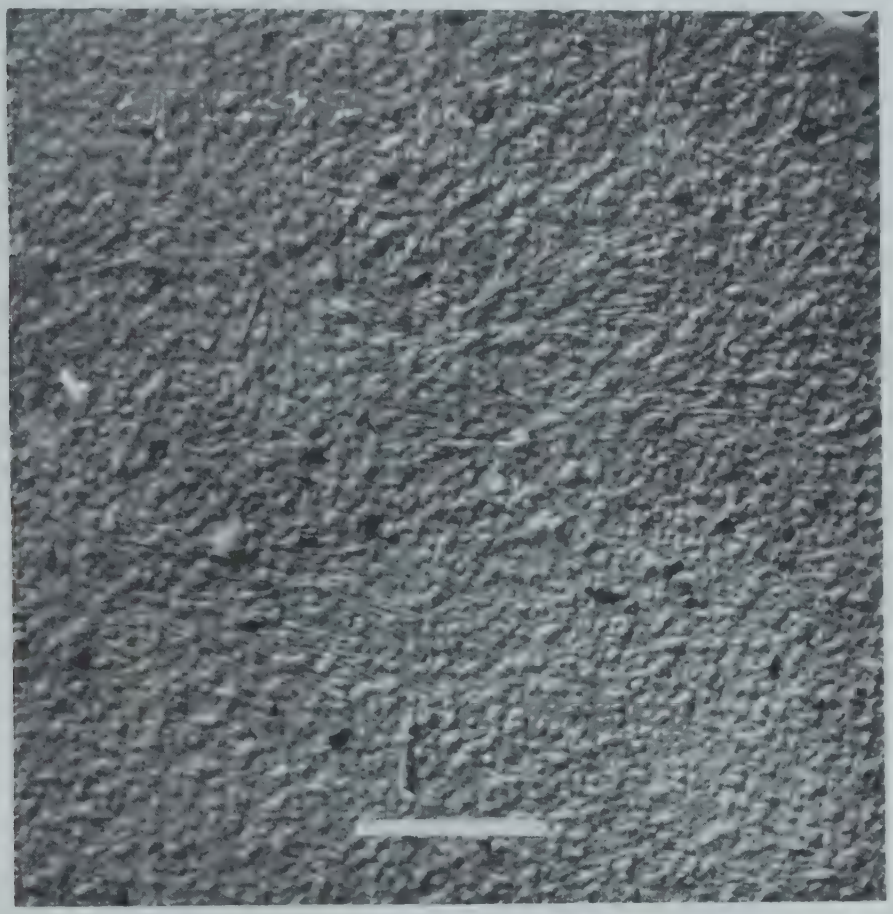


FIG. 36. EMP of fiber bundles of actin ("spherulae") in the process of formation from G-actin. (Rozsa, Szent-Györgyi, and Wyckoff, 1949.)

fixed. In Fig. 33 we see fibers of limited length growing out of one center of crystallization in the presence of a great excess of G-actin. These very thin fibers can be looked upon as monodimensional crystals forming little spherulae. If there are many such centers, the fibers are short. If there are but few centers, the fibers may grow to a great length, and all the G-actin may be built into these fibers. In this case the picture will not be obscured by the excess G-actin. Such pictures are reproduced in Figs. 37–39.

In Fig. 37 most of the fibers are seen running in a north-south direction. Where several threads come to lie side by side, they show

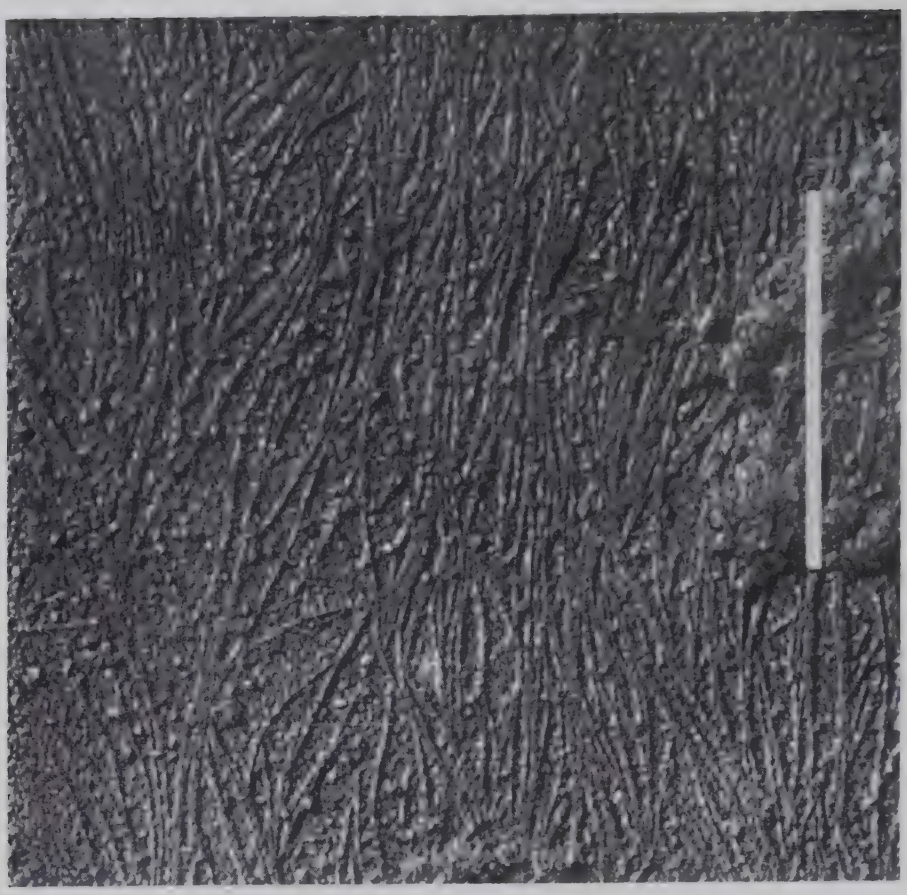


FIG. 37

FIG. 38

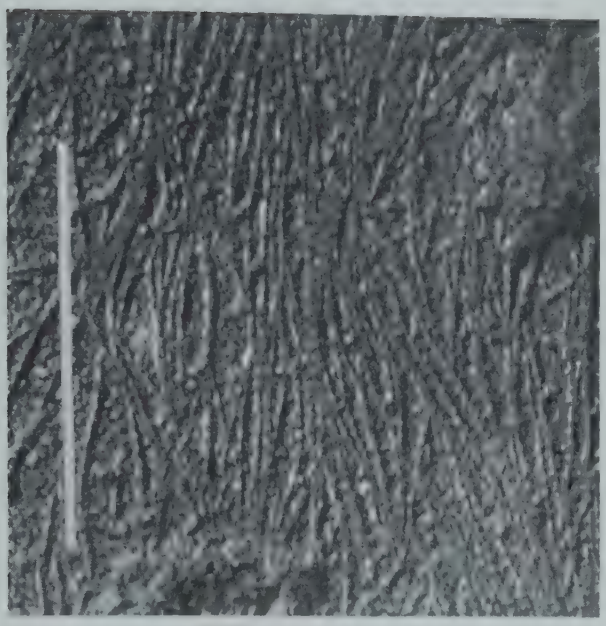


FIG. 39

FIGS. 37–39. EMP of F-actin fibers formed *in situ* from G-actin. (Rozsa, Szent-Györgyi, and Wyckoff, 1949.)

a striking new quality; a cross striation. This cross striation in Fig. 38 is so pronounced that at first glance one is at a loss as to which direction should be taken as the real axis. There is evidently no real axis, or more exactly there are two, crossing one another at a sharp angle, and so the system actually is a two-dimensional net. Figure 39, taken at the highest magnification, gives the most information about the nature of this cross striation. It shows that the single threads are built of slightly elongated particles, *ovoids*, about 3 times as long as they are wide, and if two threads come to lie side by side, this is done in such a way that the single ovoids of the two neighboring threads also come to lie side by side. The particles are thus associated both end to end, building the single fibers, as well as side to side, causing cross striation and building the single fibers into a two-dimensional network of great regularity.

This regularity of arrangement opens an important possibility: it allows us to measure the dimensions of the single ovoids. The outlines of a single particle of this size could not be seen in the EM, the particle being of the same magnitude as the roughness of the background. If, however, a regular net is formed, the spacings of this net can be seen clearly and measured with fair accuracy. The longitudinal spacing corresponds to the width of the particles, while the cross spacing corresponds to their length. The measurements show that the length is about 300 A, while the width is 100 A. Naturally, this space

cannot be filled completely by protein; if it were, this would give a MW of 2×10^6. Proteins usually bind about their equal weight of water, which gives us a MW of 10^6 g, the real MW being probably somewhat below this value. About a dozen globules of MW 70,000 must have united to form one ovoid. Globules of MW 70,000 are about 50 A in diameter, therefore an ovoid would accommodate two globules crosswise and something like half a dozen lengthwise. Astbury and Spark, with their X-rays, find a spacing of 54 A in actin (see Astbury, Perry, Reed, and Spark).

As the main result of these observations we can state that when the actin fibers are built out of globules, this is done in two stages. First, larger particles, *ovoids*, are built out of the smaller *globules;* then the ovoids are put together to form *fibers*. It seems possible that the fiber is held together by dipole forces and that the ovoids are built because only such bigger and elongated particles can develop sufficiently strong dipole moments to stand the strain to which such fibers are exposed in muscle. In tactoids with elongated particles the dipole forces are usually stronger along the axis and weaker across. Therefore we can expect the end-to-end association to be strong and can expect the system to grow lengthwise rather than crosswise and to break up rather in the latter direction.

The ovoid, with its dimensions of 100×300 A, represents an important step on the scale of organization and one wonders what are the new qualities which are linked to this level. One may find some lead in Bernal's experience (1949) collected on the tobacco mosaic virus (page 909): "Once the size of 100 angstroms or so is surpassed a new kind of interactions, imperceptible against the thermal background in smaller sizes, becomes apparent. These are long-range forces as assumed to account for many colloidal phenomena, such as gel formation and coacervation. Tobacco mosaic virus provided the first quantitative picture of such forces, because it showed that the identical rod-shaped particles of the virus maintained themselves at equilibrium distances."

All this leads us to an important conclusion. The activity of muscle must depend on the field of forces surrounding the ovoids, the forces generated by these particles. These forces, in turn, depend on the way in which the smaller globules are put together and on the forces which hold them together. It is thus not surprising to read Astbury and Spark's conclusion that "The detailed construction of the F-actin fiber X-ray diagram indicates that the corpuscular units from which the fibers are formed are not strung together in arbitrary fashion, but always in the same way and with atomic precision." It also follows

that it will not be necessary to disintegrate the ovoids as far as into globules in order to change the outer field of forces. The subtlest change inside the particle will express itself in a change of the outer field. The G-F transformation is merely the extreme case of such a change. As long as the ovoids are surrounded by a strong field of forces and are linked together by strong forces, they cannot be taken out of the edifice of the muscle fibril. It is necessary to disintegrate the ovoids into smaller globules and abolish these forces. Fortunately, the process is reversible, which opens up the unique possibility of gradually breaking down the muscle fibril, at least the actin part of it, to relatively simple and small macromolecules of MW 70,000 which, in themselves, show no more signs of life or any biological function; then we can walk back the same way, putting ovoids and fibers together again.

Actin and Ions. A solution of G-actin, as prepared by Straub's method, is free of salts and does not polymerize spontaneously. If any salt that does not cause denaturation is added to the fresh preparation in the proper concentration, the G-actin polymerizes to F-actin. This indicates that the polymerization of the globules into ovoids depends on charges which are imparted to these particles by the ions.

The G-F transformation has the character of an autocatalytic reaction. There is a shorter or longer latency period during which apparently nothing happens. Once the viscosity begins to rise, it does so rapidly until the reaction is completed. Sometimes it even overshoots the final value for a short time. The reaction is not truly autocatalytic because the addition of F-actin does not accelerate it.

There are marked differences in the activity of the various salts, showing that the ions cannot be regarded simply as charged points but that their action is specific. Not only do they act differently, but there is also a subtle interrelation between them which resembles "physiological ionic equilibration."

The most active monovalent ion is H. Actin readily polymerizes at pH 6, the length of fibers decreasing with the pH (Jakus and Hall).

The alkali metals are active in the following order: $Rb < K < Na < Li$, while the anions, if added with the same cation, are active as follows: $J < Br < Cl < F$. Consequently, the most active salt is LiF. SO_4 is less active than Cl. Evidently we are not dealing with the isolated action of anion and cation here, but, as in the case of myosin, there is an interrelation between the two.

Naturally, every salt has its optimal concentration. For NaCl or KCl this lies around the point at which it is isosmotic with blood or muscle. As we increase the concentration above this point, the rate

of polymerization falls off rapidly (Fig. 40). KI, like any other alkali salt, polymerizes in 0.1 *M* concentration, but depolymerizes F-actin instantly and irreversibly in 0.6 *M* concentration (Guba, 1950).

The relation between K and Na is remarkable. While KCl and NaCl act in the same fashion, KCl added to the optimum concentration of NaCl in the same proportion as these ions are found in blood still markedly increases the rate of polymerization.

Mg has a specific action. In its entire absence there is no polymerization at all, and if small quantities of Mg are present, the rate of polymerization in NaCl is proportional to the Mg concentration. If

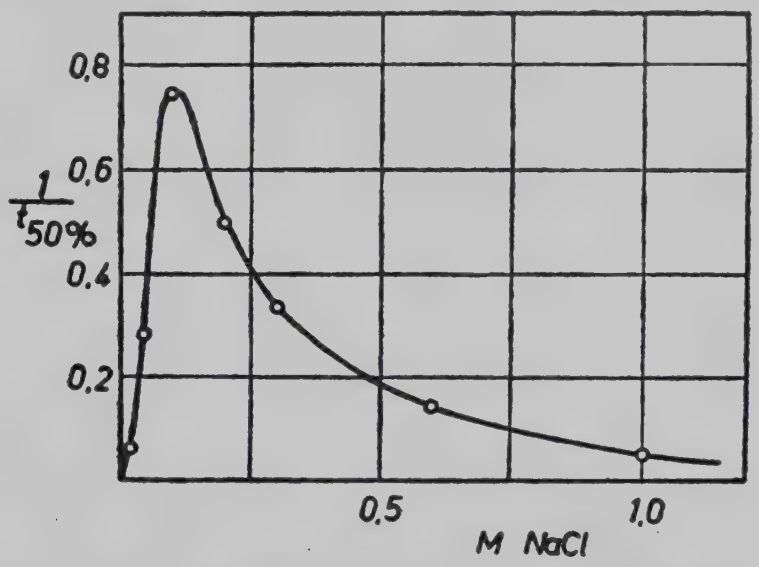


FIG. 40. The rate of polymerization of actin at varied [NaCl]. Ordinate, reciprocal value of the time in minutes for 50 per cent polymerization at 24°C. (Straub *et al.*, 1948.)

actin polymerizes in NaCl without the addition of Mg, this is because actin preparations always contain Mg. This activating effect of Mg is produced by a concentration as small as 0.0005 *M*, insufficient in itself to induce polymerization. At higher concentrations polymerization is induced by $MgCl_2$ alone. If the Mg is bound by Calgon, the actin no longer polymerizes. but if the protein is incubated with Mg, and Calgon is added afterward, the actin will polymerize on the addition of NaCl, which may equally mean that Mg does something to the actin which enables it to polymerize, or that Mg on incubation becomes inaccessible to the Calgon. The action of Mg consists of reducing the lag period rather than of increasing the rate of polymerization. In the absence of Mg, F-actin loses thixotropy.

The relations between actin and Ca are rather intimate and complex. Actin, as we know it, is a Ca actinate. However carefully

washed, the actin preparation always contains about four atoms of Ca per molecule (0.215%). There is thus one Ca for every 18,000 g. very firmly bound. What actin would be like without this Ca we do not know; probably it would not be actin at all. But apart from actually being part of the molecule, Ca has an additional great affinity to actin and precipitates it in 0.002 *M* concentration quantitatively. Much higher concentrations of Mg are needed to induce the same effect, and alkali metals do not precipitate it at all. The Ca precipitate according to Erdös (I) contains ten equivalents of Ca for every 18,000 g.

Between Ca and alkali metals there is a remarkable "equilibration." Ca, which in itself can induce polymerization, retards the same process induced by NaCl, increasing the lag period.

The polymerization induced by salts is reversible. If a filter paper is covered by a thin sheet of colloidal caolin in the Buchner funnel, it will let the G-actin through, but will retain the actin if it is in the F-form. If all the fluid is sucked through and the F-actin is washed with water, it readily depolymerizes and passes through the filter.

G-actin can also be brought to polymerization by freezing, probably owing to the concentration of the salts present. When thawed, the actin is found in the F-form, which will then depolymerize again spontaneously. There is thus an equilibrium between particles and the ionic atmosphere, and any change in the ionic milieu will change the forces holding the globules together and will change their interrelation within the ovoid and thereby change the outward forces developed by the latter. This will also alter the forces developed by the muscle in contraction. Changes in the rate of polymerization are a good indicator of these relations.

Straub, Feuer, and Lajos also found that drugs known to have an effect on the function of muscle *in vivo* also modify polymerization rates of actin. Adrenalin, known to increase the activity of fatigued muscle, increases the rate of polymerization, the effect depending on ionic equilibration and being the highest at a Na : Ca ratio, which corresponds to the ratio of these ions in blood. Strychnine, quinine, and veratrine had an inhibitory action, while cholin had none. The concentrations in which all these drugs were employed were rather high, which sheds some doubt on the specificity of the effects observed.

Actin and ATP. Straub (1950) and his associates made the discovery that ATP is needed for the polymerization of actin. Preparations of G-actin always contain 1–1½ molecules of ATP per molecule of actin. On storage of the G-actin solution, this ATP is decomposed and the actin loses its polymerizability. If the ATP is eliminated in

any way, be it by dialysis, enzymatically, or by isoelectric precipitation, the actin no longer polymerizes and soon denatures. The presence of ATP seems to be a *sine qua non* of stability and without it, actin is not actin and would not polymerize. Myosin has also demonstrated its dependence on the presence of ATP for stability; but the effect was less striking. Laki, Bowen, and Clark, also arrived at a similar conclusion. They were able to demonstrate the stabilizing effect of ATP in a rather dramatic fashion by adding a small amount of $(NH_4)_2SO_4$ to the G-actin solution. If ATP was present, it induced polymerization; if none was present, it induced denaturation and precipitation. In the absence of Mg, there was neither polymerization nor precipitation. Once the F-actin has been formed, it remains good F-actin even without ATP. The forces leading to denaturation seem to be depolarized both by ATP and by the association of the actin globules.

In a later paper, Straub and Feuer (1950) extended these observations with experiments, which, if corroborated, could not fail to have a profound effect on our whole concept of muscular activity and energy coupling. They found that ATP was dephosphorylated during polymerization to ADP, the two changes running parallel. Depolymerization restored the ATP again. Their final conclusion was that G-actin is ATP actin, while F-actin is ADP actin, and they believe that this change is the key to the understanding of energy coupling and the contraction cycle. They call ATP and ADP the prosthetic group of actin.

These conclusions are borne out in part only by experiments of the author's associates. There seems to be a relation between polymerization of actin and the dephosphorylation of ATP but hitherto no evidence could be found for the reversal of the process, that is the phosphorylation of ADP during the depolymerization of actin. Laki, Bowen, and Clark could separate ATP from actin by isoelectric precipitation of the protein, a behavior not expected from a prosthetic group. The same workers find Straub's actin rather inhomogeneous, find more adenine in their preparations than corresponds to the ATP content and find indications that besides ATP other factors are also involved in polymerization.[3]

Dubuisson (1950c) could find no relation between actin polymerization and dephosphorylation. Evidently conditions are complex and the final judgment on the role of ATP in actin polymerization must

[3] The polymerizability could be restored to the actin by the addition of ATP only if the ATP was eliminated enzymatically (by hexokinase plus hexose or phosphorpherase plus creatine), but not if the ATP was removed by isoelectric precipitation.

be reserved till more experimental material accumulates. At the moment it seems probable that the energy of the P—O—P bond of ATP is utilized to establish a bond between acton globules and ovoids and that the active points, thus linked together, have to be kept depolarized by ATP in G-actin, or else the molecule deteriorates.

Oxidation of Actin. As observed by Feuer, Molnár, Pettko, and Straub, actin contains easily oxidizable groups the presence of which is a prerequisite of polymerization, which process can be halted by oxidizing these reducing groups by $KMnO_4$. If small quantities of this oxidant are added, the effect is reversible and polymerizability can be restored by ascorbic acid or cysteine. Much $KMnO_4$ destroys the actin altogether. Small concentrations of methylene blue or cysteine also cause reversible oxidation, whereas ferricyanide or quinone have to be used in higher concentrations. The reversibly oxidized product is very unstable and deteriorates rapidly at 40°C. The viscosity of F-actin drops when $KMnO_4$ is added, indicating a breaking up of the fibers into shorter fragments. The oxidative degradation proceeded stepwise in three phases.

Actin and Myosin. As observed by the author, myosin has a remarkable catalytic effect on the polymerization of actin. In the presence of myosin, at a low salt concentration (0.1 *M* KCl), the polymerization of actin is instantaneous. At least this is true at room temperature, but the reaction has a very high Q_{10} and proceeds rather slowly around 0°C. In the presence of high salt concentrations (0.6 *M* KCl), myosin has no enhancing effect at all. If anything, its effect is inhibitory, which may be due to the decomposition of the ATP present. The myosin catalysis of the G-F transformation is very striking and cannot fail to have its bearing on the muscle in which actin and myosin are in closest proximity.

Chapter VIII

Actomyosin

1. F-Actomyosin

If F-actin and myosin are mixed in a solution, a sudden change in the physical properties indicates that a reaction has taken place and a complex, "actomyosin," has been formed: there is a strong increase in light scattering and double refraction of flow, and even at low velocity gradients the particles will readily orient themselves parallel to the wall (Mommaerts, 1945). There is a strong rise in viscosity (Fig. 20), which is not only high, but also strongly anomalous. If the protein concentration is high enough, the mixture turns into an elastic gel. The actomyosin, dissolved in 0.6 *M* KCl, is moderately thixotropic.

The rise in viscosity is rather unexpected. F-actin consists of very long filaments in any case, and therefore the rise in viscosity could hardly be due to the formation of still longer threads. The filaments must have become more rigid, or must have formed some sort of superstructure, an anastomosing network. As will be seen later, even a dilute actomyosin solution behaves as a continuous gel. The EM pictures (Reed, Astbury, and Spark) also support this idea.

In a way, actomyosin is a stoichiometric compound. Straub (1943) has found that actomyosin shows the strongest physical change when ATP is added if it contains actin and myosin in the ratio 1:2.5, the same ratio in which the two proteins are contained in muscle (Balenovic and Straub). Snellman and Tenow (1949) found that actin in the ultracentrifuge carried down constant quantities of myosin, 2.5–3 times its own weight. Weber (1950) reports the same experience. Gergely and Spicer found that if actin and myosin were mixed in various proportions, there was one in which the two proteins precipitated one another quantitatively when ATP was added. The privileged ratio was found to be 1:2.5 if the total protein content of the "actin" solution was taken into account. If one of the two components was in excess, some of it was left in solution and some of it was taken down, and the

precipitate was more bulky. Mihályi (oral communication), in his first tentative measurement of the acid titration curve of actin, found that this protein has three times as many dissociable groups as myosin, thus giving an adequate explanation for the 1:3 ratio. Lately some doubt has been shed on this quotient by Laki, Bowen, and Spicer (oral communication) who found G-actin dishomogeneous. Nevertheless, at the moment it seems that doubtless there is some preferred ratio, and it seems probable that this actually is about 1:3.

The properties of actomyosin suggest an electrostatic bond. If the KCl concentration is increased above 0.6 *M*, the viscosity drops, to reach the additive value of actin and myosin asymptotically at 2 *M*, suggesting that the complex has dissociated into its components. At a low temperature (0°C) and a high salt concentration (0.6 *M* KCl) very small concentrations of polyphosphates (all of which are polyvalent anions), be it ATP, inorganic pyro- or triphosphate, cause immediate dissociation of the two proteins.

Bailey and Perry have developed a theory which makes the SH groups of myosin responsible for both the ATP-ase activity and the binding of actin. According to this theory, ATP dissociates actomyosin because it competes with actin for the SH, which, at the same time, is also the active group of the ATP-ase. It is regrettable that the experiment does not support this ingenious theory which was based chiefly on the fact that intact SH is a prerequisite of both ATP-ase activity and actomyosin formation. If the theory were correct, then actomyosin should not be ATP-ase at all, its SH being blocked. The fact is that it is a most active one. Moreover, SH is not known to have a specific affinity to polyphosphates. Guba (personal communication) has shown that four-fifths of the SH groups of myosin remain accessible to bromoacetophenon in actomyosin. Gilmour and Calby's experience with ninhydrin did not favor the theory either. Certainly, SH is needed for association of actin and myosin, but so is Mg (in the absence of which actomyosin is unstable and dissociates spontaneously in 0.6 *M* KCl), and so are the reducing groups of actin (Feuer, Molnár, Pettko, and Straub). Banga (I) found a definite increase in pH during actomyosin formation, which also is not explained by the SH theory.

The colloidal behavior of actomyosin reflects the behavior of myosin, but the reactions are made rather sluggish by the presence of actin. In the entire absence of salts an actomyosin gel swells to a glassy mass and may dissolve by endless swelling; 0.1 *M* precipitates it (at pH 7) and higher salt concentrations, exceeding 0.4 *M* (KCl or NaCl) redissolve the precipitate.

The same sluggishness is also found in relation to pH. Like myosin,

actomyosin is also precipitated by acid isoelectrically, but instead of an "isoelectric point" there is an "isoelectric zone" extending over 2 pH units which, as shown by Sarkar (Fig. 41), is shifted by small [KCl] to the alkaline and by big [KCl] to the acid side, the "precipitation by salt" being merely a shift of the IP here also.

Since both actin and myosin are precipitated by Ca and Mg, actomyosin is also precipitated by them.

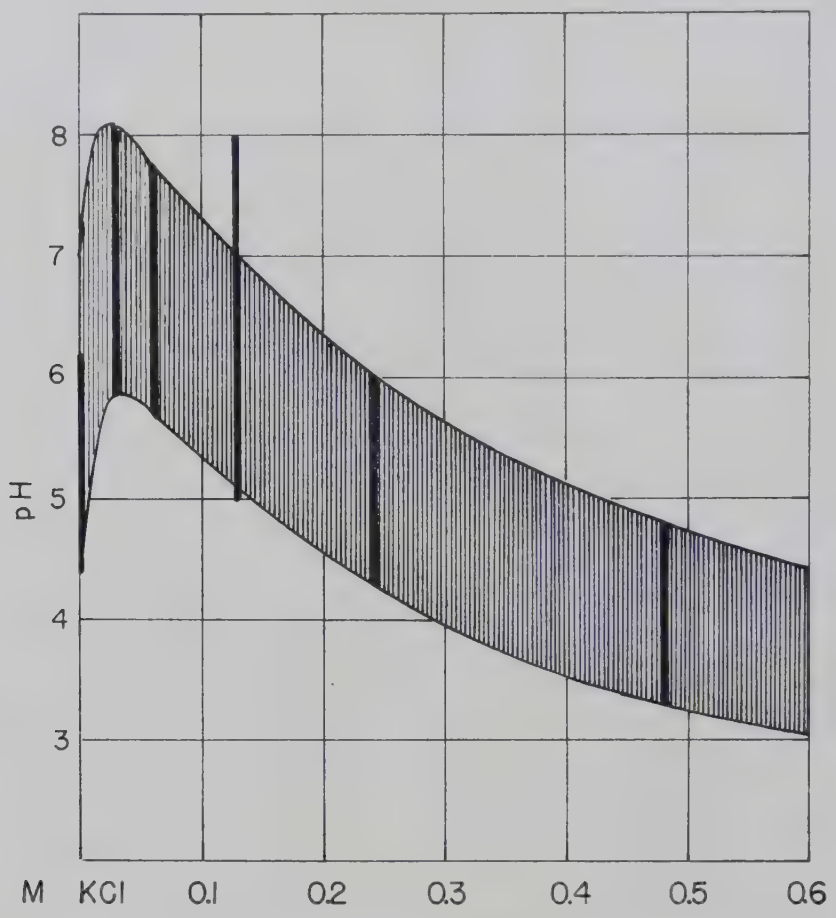


FIG. 41. Zone of isoelectric precipitation of actomyosin at varied [KCl]. Thick lines, same in the presence of ATP. (Sarkar.)

As in the case of myosin, precipitation by salts does not mean an inner discharge of the particle, but merely the depolarization of the over-all charge. The actomyosin, at the maximum of its precipitation by 0.1 *M* KCl, is still very hydrophylous and precipitates in the form of loose, strongly hydrated floccules.

Before leaving actomyosin, mention should be made of one of its properties which is useful sometimes and unpleasant at others: *hysteresis.* A freshly prepared actomyosin thread is rather soft and fragile and redissolves readily in 0.6 *M* KCl, but if it is stored for an

hour or so, it becomes rather elastic and may not dissolve even in 1.0 *M* KCl. If such a thread is stretched and released, it snaps back to its original length, but if kept in a stretched condition for some time it "sets." A freshly prepared actomyosin can easily be dissociated again into actin and myosin, but if the compound has been stored for some time, it will demand a more drastic procedure to produce dissociation. The more discharged the condition of the actomyosin the stronger this hysteresis will be.

2. F-Actomyosin-ATP

The reader must have found actomyosin rather disappointing. We had two most fascinating highly reactive proteins, actin and myosin.

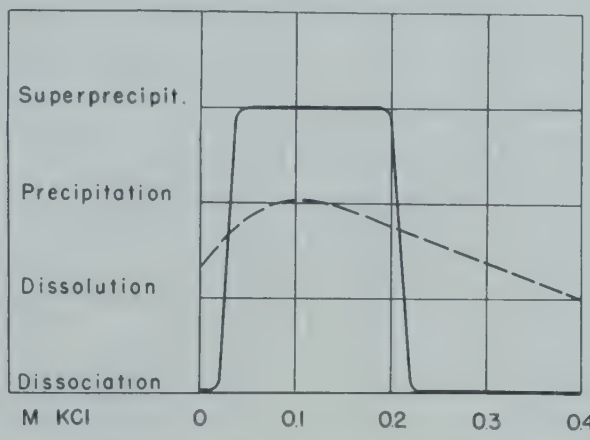


Fig. 42. Schematic representation of behavior of actomyosin at varied [KCl] in the absence (broken line) and presence (full line) of ATP.

We put them together, taking one more step toward muscle, and produced a dull, clumsy, sluggish compound with none of the wonderful responsiveness of muscle. The reader will now be compensated by our introduction of ATP into the system, which makes the dull story dramatic, reactions violent, changes abrupt and excessive. This is shown schematically in Fig. 42, where the broken line represents the behavior of actomyosin in the absence of ATP, the full line in its presence. In the absence of ATP, when the KCl concentration is increased, there is a gradual and moderate precipitation, followed by a gradual dissolution and dissociation. In the presence of ATP (0.05%), at a low salt concentration, there is a complete dissolution and dissociation (full line in Fig. 42). Then, when we gradually increase the [KCl], suddenly we find not only precipitation but also an exceedingly intense one. Instead of loose, bulky floccules, a dry-looking, granular "superprecipitate" is formed which settles rapidly to the bottom of the test tube, occupying a

small volume there [Fig. 23(*a*)]. As we increase the [KCl] further,[1] suddenly complete dissolution and dissociation take place again. If two consecutive test tubes differ by no more than 0.02 *M* KCl, in one we may find the actomyosin superprecipitated, in the next dissolved and dissociated. That the complex actually dissociates is shown by its limpidness and low viscosity. The transition is abrupt, all-or-none, with nothing between. That dissolution actually means dissociation was also shown in the EM by Perry, Reed, Astbury, and Spark and in the ultracentrifuge and Tiselius (where the dissociated components can be readily separated) by Snellman and Erdös as well as Johnson and Landolt; Weber; Snellman, and Gelotte.[2]

If superprecipitation is elicited in an actomyosin "solution" in which the continuity of the actomyosin network was not broken up by agitation, instead of forming a granular precipitate, the actomyosin contracts to a plug [Fig. 23 (*d*)]. If the same reaction is produced in a gel and ATP is added to an actomyosin thread, excessive and rapid shrinking is obtained, as discussed earlier (Chapter V).

The critical concentration of KCl, at which superprecipitation yields to dissociation, also depends on the ATP concentration. More ATP makes the zone of superprecipitation smaller. The same is true for Mg (0.001 *M* $MgCl_2$), which greatly increases the affinity of myosin to ATP, and thus acts as a high ATP concentration narrowing down the zone of superprecipitation to the range of 0.05–0.12 *M* KCl, but making the superprecipitation within this narrow zone very intense. Mg also greatly speeds up the reaction, which is the strongest if both K and Mg are present (Erdös, 1941–42).

Superprecipitation also depends on temperature. At 0°C there is no superprecipitation. As the temperature is increased, the superprecipitation appears to increase with the temperature and become maximal around 15°C.[3] Although there is no superprecipitation at 0°C, acto-

[1] The rate of superprecipitation is the greatest at the KCl concentration which causes maximal precipitation in itself (0.1 *M*). At higher salt concentrations superprecipitation is maximal, but its rate may be slower.

[2] Jordan and Osler from measurements of light scattering make the coiling of particles responsible for the drop in viscosity. Their result is rejected by Johnson and Landolt as well as by Mommaerts (personal communication). The great bulk of evidence available now leaves little doubt about the dissociation of the compound under the influence of ATP. The breaking down of the superstructure may have been responsible for Jordan and Osler's results. Their EMP's, adduced as evidence, were too vague to admit any conclusion.

[3] Precipitation itself is not a true measure of the reaction, because, if incomplete, the unprecipitated particles may protect the discharged ones from flocculation. The dimensional change in a gel is a better indicator.

myosin is nevertheless formed, as indicated by the increasing light scattering and viscosity. We can thus state that superprecipitation takes place in two stages. First an unprecipitated (uncontracted, relaxed) actomyosin is formed. This reaction is not dependent on temperature and takes place even at −4°C, (B. Horváth, personal communication). Then a second reaction takes place within the complex, which expresses itself to the observer as superprecipitation. This reaction is reversible, especially if hysteresis is inhibited by a higher pH. As shown by Gergely and Spicer (1950b) at pH 8 or slightly above (8.3), the superprecipitate dissolves when cooled and forms again when the fluid is warmed. The whole phenomenon is very similar to the reversible heat denaturation of the trypsin-inhibiting factor of M. Kunitz. If we like, we can call superprecipitation a reversible heat denaturation.

This temperature dependence of superprecipitation explains the abruptness of the transition from superprecipitation to dissociation at room temperature.

The chain of events was as follows:

$$\text{F-actin} + \text{myosin-ATP} \rightleftharpoons \text{F-actomyosin-ATP} \quad \text{(unprecipitated)} \rightleftharpoons \text{F-actomyosin-ATP} \quad \text{(superprecipitated)}$$

Superprecipitation being complete at 15°C or above, there can be no unprecipitated actomyosin at room temperature. This state therefore drops out:

$$\text{F-actin} + \text{myosin-ATP} \rightleftharpoons \text{F-actomyosin-ATP} \quad \text{(superprecipitated)}$$

We have thus, apparently, a direct transition from the dissociated into the superprecipitated state, the complex being either superprecipitated or dissociated, the transitory state, unprecipitated actomyosin being unstable above 15°C. If at room temperature there is no superprecipitation—either ATP or actomyosin must be absent.

The point of transition between the two states, superprecipitation and dissociation, depends on various factors. Starting from the maximum of superprecipitation, increased [KCl], [Mg], [ATP], pH, and low temperature will favor dissociation, and the opposite will favor association. This is nicely demonstrated by the experimental Table I in which a series of test tubes was filled with an actomyosin-ATP mixture, the KCl concentration being varied from tube to tube. After the first minute superprecipitation began only with the lowest KCl. As the ATP concentration was gradually reduced by its splitting, the superprecipitation spread to the higher KCl.

TABLE I. Superprecipitation of actomyosin at varied KCl concentrations.

	M KCl								
Minutes	0.100	0.106	0.112	0.118	0.124	0.130	0.136	0.142	0.148
1	X	—	—	—	—	—	—	—	—
2	XXX	X	—	—	—	—	—	—	—
3	XXX	XXX	XX	X	—	—	—	—	—
5	XXX	XXX	XXX	XXX	X	—	—	—	—
15	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	X

X = superprecipitation (I).

In describing superprecipitation we must be careful with our wording. This is a reaction produced within the system, consisting of myosin, actin, ATP, and salts; if any of these is missing, superprecipitation does not take place. Superprecipitation will thus apparently always be elicited by the substance which was added last and which completed the system. We can say that the "ATP causes actomyosin to superprecipitate in the presence of KCl" or "KCl superprecipitates actomyosin in the presence of ATP," or "actin induces superprecipitation in myosin in the presence of KCl and ATP."

Of the two reactions, dissociation and superprecipitation, the former is the easier to explain. Obviously there must be an electrostatic repulsion between actin and myosin to separate them. Actin is more negative than myosin, its IP being at a lower pH, therefore we can expect that any influence which decreases the negative charge of myosin will tend to bring the two colloids together, while an increase of the negative charge will push them apart. As Fig. 30 showed, KCl charges myosin negatively above 0.22 *M* and does the same above 0.12 *M* in the presence of Mg. ATP will cause dissociation of actomyosin, above 0.22 *M* KCl in the absence, above 0.12 *M* KCl in the presence of Mg (0.001 *M*). This effect of ATP being a question of charge is not specific and is duplicated by inorganic pyrophosphate (Straub, 1943) or triphosphate at 0°C.

The difficulty is with the interpretation of superprecipitation. *If* the coulombic forces do not push actin and myosin apart and the two colloids unite in the presence of ATP, a reaction occurs which does not occur in its absence, which declares itself in a complete discharge entailing a loss of affinity to water. While an actomysin thread usually contains 2–3 per cent protein and 97–98 per cent water, even though it consists of actomyosin precipitated by KCl, if ATP is added and the thread contracts, in its contracted state it will contain 50 per cent protein and 50 per cent water and will thus consist of practically "dry" actomyosin, the 50 per cent water being there merely because closer packing of a protein is hardly possible. Evidently, in the presence of

ATP not only was the over-all charge neutralized, as was the case when KCl precipitated myosin or actomyosin in the absence of ATP, but the violent precipitation and complete dehydration in the presence of ATP also indicates that the discharge went much deeper, and the inner system of charges was depolarized, to which the colloid owed its great hydrophility.

At the moment we can only speculate upon the role played by ATP in this reaction. It is possible to believe with H. H. Weber (personal communication), that actin and myosin would also discharge one another in the absence of ATP if the reaction did not stop at a false equilibrium for steric reasons and the rigidity of the particles. ATP helps the system to reach true equilibrium, acting, so to speak, as a lubricant. This explanation is supported by the fact that superprecipitation is the strongest at salt concentrations which, in themselves, precipitate the strongest, so ATP merely intensifies this salt precipitation. At the same time it is somewhat difficult to see, if this were all, how the energy of the terminal phosphate of ATP could pay for the energy expenditure of contraction and how ATP could bring back the system to relaxation and dissociation, as it does in muscle. Another explanation would be to suppose that ATP by being adsorbed at a strategic point of myosin (or giving its terminal PO_4 to the myosin at this point) would change the pattern of charges in myosin in such a way that they could now neutralize the charges of actin. Another explanation might be that ATP brings about an endergonic synthesis of actin and myosin, producing a "high-energy link" between the two. Then the mutual discharge of actin and myosin would spread from this point by a "zipper action." In all these cases we have described superprecipitation as the mutual discharge of two proteins. We must not forget, however, that the discharge also depends on ions which may be responsible not only for establishing the necessary pattern of charges. These are all possibilities and there are many others. A wealth of possibilities shows poverty of knowledge, and so for the time being we had better be content merely to note the fact that when actin and myosin come together in the presence of ATP, an extensive discharge occurs with all its consequences, dehydration, precipitation, or shrinking, and the role played here by ATP is not understood. The over-all charge is not changed by ATP in this process, as shown by the thick lines in Fig. 41, which denote the zone of isoelectric precipitation of actomyosin in the presence of ATP, which changed the isoelectric precipitation into a superprecipitation.

In 0.6 *M* KCl, in the absence of ATP, actomyosin is on the verge of dissociation. As the decreasing viscosity shows (Fig. 42) it has

already started to dissociate. It thus takes very little ATP to make the dissociation complete. According to Mommaerts (1950*) 1 mole ATP per 300,000 g actomyosin is sufficient. To induce maximal superprecipitation or contraction, much higher ATP concentrations are needed; these changes become maximal at an ATP concentration at which 4 moles of ATP are adsorbed by 70,000 g of myosin present, that is, 1 mole of ATP by every 18,000 g myosin.

In conclusion one more observation may be mentioned: Borbiro (unpublished) has shown that similarly to superprecipitation, the simple salt precipitation of actomyosin has a positive temperature quotient, while the salt precipitation of myosin shows no definite temperature dependence. This is one more instance of ATP merely promoting a process which would have happened to a minor degree in its absence. It is also worth noting that the effect of Ca on superprecipitation is antagonistic to that of Mg, and 0.01 *M* $CaCl_2$, added to the 0.1 *M* NaCl, completely inhibits superprecipitation. The addition of Mg, in an equivalent quantity, breaks through the inhibition and produces superprecipitation in spite of the presence of Ca.

Summing up, we might say that if actin and myosin are brought together in the presence of ATP and ions, one of two things may happen. If the repulsive charges between the two proteins exceed a critical level, the two, actin and myosin-ATP, are driven apart. If the repulsive charges drop below a critical level, the two unite to form actomyosin and a reaction takes place within the complex entailing the collapse of the involved system of charges. The critical charge depends on the ionic atmosphere in a subtle way. Working in KCl, a difference in concentration of 0.02 *M* may suffice to bring the superprecipitated system to dissociation and vice versa, clearly showing the electrostatic character of the primary actin-myosin link.

ATP itself has a double function, a static and a dynamic one. ATP keeps actin and myosin apart in the state of rest and makes the system elastic and supple, decreasing cohesive forces. This elasticity is not necessarily an expression of the dissociated state of actomyosin. ATP also makes glycerinated psoas fibers elastic, though actin and myosin are permanently united in this material. The dynamic function of ATP consists of inducing contraction. External work being done in this function, it entails the release of chemical energy and the splitting of the high-energy P–O–P link.

In resting muscle, well provided with ATP, there are 3 to 4 moles of this nucleotide bound to every 70,000 g of myosin, one of which will only be split eventually in contraction to restore the energy balance. This may mean that one of the 4 ATP molecules, linked to

the 70,000 g unit, partakes actually in contraction, and the others fulfil only a static function. It may, however, also mean that all of the ATP's partake in the mechanism of contraction, but that the system pays for the energy expense by eventually sacrificing one of its P–O–P links. This possibility is supported by the observation (Borbiro) that the whole ATP concentration is needed to develop maximal tension.

The actions of ions, discussed in this chapter, are all reversible. Reactions with heavy metals are different. Only Cu has hitherto been studied, this ion being an important constituent of "distilled water." It acts on "myosin" in three stages. In the first, the action of Cu is indicated only by the failure of "myosin" threads to contract on addition of ATP. This action can be reverted by eliminating the Cu by KCN. If the action of the metal lasts longer it catalyzes the autooxidation of the reducing groups present. In order to elicit contraction now it is not sufficient to treat the thread with dilute KCN; it will contract only if, in addition, it is also treated with cysteine which again reduces it. In the third stage, the protein denatures and becomes highly elastic. It can be stretched now reversibly by 300 per cent whereby it develops a strong double refraction. Such threads are an excellent material for the study of certain physical properties of myosin. The change is irreversible. Weber's fascinating studies on actomyosin threads must have been done on such Cu-denatured material, no attention having been payed to the purity of the distilled water. The author's early studies with Gerendás (1940) were also made on Cu-myosinate.

Since all known function of myosin is dependent on the presence of intact SH groups, it is not surprising that there is no superprecipitation (Godeaux) or contraction without them.

3. G-Actomyosin and G-Actomyosin-ATP

Very little is known about the properties of G-actomyosin. If G-actin and myosin are mixed at room temperature at a salt concentration which is not far from isotonic (0.1–0.2 *M* KCl), the myosin greatly catalyzes the G-F transformation and the G-actin is transformed into F-actin instantaneously, so that F-actomyosin is obtained. If the concentration of the proteins is not too low, the whole fluid may turn into a gel. At higher salt concentration, at 0.4 *M* KCl or above, there is no change in viscosity when G-actin is added. At these higher salt concentrations myosin does not catalyze the G-F transformation and even the spontaneous transformation of G-actin seems to be slowed down, which may perhaps be due to the myosin decomposing the present ATP. Whether the G-actin unites with myosin at these

higher salt concentrations or not cannot be stated. If it does, this does not alter the viscosity of myosin.

If G-actin is added to myosin in the presence of ATP at a lower salt concentration, the two proteins unite to form G-actomyosin. There is no time for the G-actin to polymerize to F-actin, because the G-actomyosin superprecipitates to form a very fine colloidal superprecipitate which is stable in this colloidal dispersion [Spicer, personal communication, Fig. 23 (*b*)]. At a somewhat higher salt concentration (0.2 *M* KCl in the absence, 0.12 *M* KCl in the presence of Mg), ATP will prevent the union of G-actin and myosin, and the myosin will not catalyze the G-F transformation. The free G-actin will polymerize spontaneously at its own slower rate. The F-actin thus formed will unite with myosin and superprecipitate under the influence of ATP, forming the usual superprecipitate if the salt concentration is not too high. At a higher salt concentration the F-actin will not unite with myosin at all while free ATP is present.

Actin and Myosin in Muscle

It is not easy to analyze muscle extracts. It is more difficult still to analyze muscle itself. But we encounter the greatest difficulty when trying to coordinate the two, the experience collected *in vitro* and conditions *in vivo*. There is relatively little difficulty with actin, which forms threads with a well-defined periodicity. There can be little doubt that the "filaments" in muscle are actually actin threads while the tactoid seen in the EMP of muscle is the three-dimensional system of these filaments.

The trouble is with myosin, which probably also consists of long threads, too thin to be resolved under the EM; so anything we say about it is more or less conjecture. One can only state which assumption, in one's opinion, is in best accord with present observations.

Let us return for an instant to Fig. 5, the EMP of a teased "muscle fibril." As will be remembered, there are two Z-membranes in this picture, lying at a relative distance of 2000 A. In the middle, between the two Z's, is the M-membrane with the thinner dark H-band and a lighter and wider "A-segment" on either side. These three together, M, H, and A, form the Q-disk, which occupies about half of the sarcomer, the part of the muscle fiber enclosed by two Z-membranes. Between the Q's stretch the I-bands with the Z-membrane in their middle.

On closer inspection of Fig. 5 we can see the filaments running right through I, A, H, and M, from Z to Z. It is possible to count how many of them fall to one definite cross section. One finds that their number is the same in all the bands. This is rather surprising because the A-band contains more solid matter as is shown by its elevation.

If we compare Fig. 5 with Fig. 6 in which the lighter middle part corresponds to the A-segment in Fig. 5, we will find that the crosswise periodicity is the same in both pictures, which means that the "filaments" seen in Fig. 5 correspond to one row of "ovoids" in Fig. 6. Thus

if the A-segment contains more solid matter than I, then it must contain some matter in addition to the filaments and ovoids, a matter which is composed of particles that are too small or too thin to be resolved by the EM.

By taking everything together, the most probable assumption at present seems to be that the "filaments" are single rows of actin ovoids. Myosin, consisting of very thin and long threads, is located in the A-band between these filaments and is invisible in the EM preparation; at the same time we owe to it the double refraction and the greater density of the structure which helps to preserve the three-dimensional regularity of ovoids. The H- and I-bands are much looser; since the space between filaments is not filled, the regularity of the arrangement of the ovoids is more easily disturbed.

The A-bands in Fig. 5, each occupy about one-third of the Q-band and have a diameter of about 3500 A. It seems probable that the single myosin particles are actually this length. Weber finds the myosin molecules about 2300 A long, 30 per cent shorter. A difference of 30 per cent in this field actually is a good accord.

These assumptions also agree with H. H. Weber's opinion that myosin is located in the Q-band only. They also agree with the contention that only the Q-band is contractile (Buchthal and Lindhard).

There is only one major difficulty in accepting these ideas. Even intact muscle can contract by 50 per cent. If the A-bands make up one-half of the sarcomer, they would have to disappear altogether to make the muscle shorter by 50 per cent. If the A-bands themselves contract by two-thirds, by 60 per cent, this would make the total length of the fiber only 30 per cent shorter. This contradiction could be bridged by supposing that the muscle, with its normal mechanism, can contract by only 30 per cent. If it shortens beyond this point, it happens with a different mechanism. This assumption can be supported by different arguments and observations. Cross-striated muscle, under normal conditions, never shortens by more than 30 per cent, usually by no more than 15 per cent. Ramsay and Street found that the first 30 per cent of shortening is different in nature from the shortening which follows, which they call the delta state. Contraction is completely reversible and is followed by active relaxation only up to this state. The glycerinated psoas, if acted upon by ATP, shortens by 80 per cent. At this degree of shortening, under the microscope, one finds the fibers broken, curved, and thrown into disorder. Evidently this excess shortening is due to some unphysiological process

and is probably caused by syneresis, the secondary consequence of the loss of charge. Such a high degree of shortening and destruction is not seen in single isolated fibers and is evidently an artifact; it is easy to believe that shortening beyond 30 per cent is already due to some unphysiological though less destructive mechanism. Muscle, if kept in this highly contracted and discharged state, may press out 35 per cent of its water, as is the case in contracture elicited by freezing and subsequent thawing in the frog sartorius.

A rather fascinating numerical relation emerges from these assumptions. Evidence will be produced in the next chapter to show that the myosin is built up of units reacting independently of one another in contraction. The MW of these units was found to be 70,000 g. If the MW of myosin is 840,000 g, then this molecule is built of 12 thermodynamic units which we will call "autones." If the whole myosin molecule reaching across the A-band is 3500 A long, then one autone is 3500:12 = round and 300 A long. This is fascinating, because 300 A is the length of the actin ovoid and means that our thermodynamic unit is simply the stretch of the myosin molecule which lies alongside and reacts with one actin particle.

If the actin filament runs continuously from Z- to Z-membrane and is accompanied by the myosin filaments, in the A-band only, then only half the actin filament could take part in contraction, while the other half would merely act, so to speak, as a tendon for the contractile part. Since the function of the two parts is different, it is credible that there are two different sorts of actin, one which builds the active and one which builds the passive part. Laki and Clark found G-actin to be heterogeneous, found it to be composed of about equal parts of two different substances which must have been closely related, since they were capable of copolymerization and of building one actin thread together (personal communication).

One may wonder about the meaning of this arrangement. Preliminary studies on the *Limulus* muscle show that in isometric contraction the Q-band shortens by about 50 per cent and stretches the I-band. This would mean that the actin filament, in the I-band, is an elastic element shunted in series with the contractile matter. This agrees also with Buchthal and Lindhard's observations.

Muscle contains about 8 per cent myosin. If this is all located in the A-band, then its concentration must approach 20 per cent. In addition to this, the A-band also contains actin and possibly also shares some of the myogen. All this together must give a rather high

protein concentration, a very closely packed structure with relatively little free water.

Naturally, this will all remain but a conjecture, a more or less probable assumption, until myosin and the A-band are resolved in the EM.

Chapter X

Thermodynamics

1. Introduction

To the physiologist muscle is a unit, and "the single twitch is the elementary unit of all muscle response" (A. V. Hill, 1949a) which he tries to study in undamaged muscle and under conditions in which Nature has intended them to work, measuring changes in shape, tension, potential, heat, optical properties, etc. These have been extensively studied during the last century, leaving behind an enormous bulk of literature and little understanding. It is difficult to read a book without cutting the leaves.

To the chemical biologist muscle is merely an involved system, and the twitch is the visible result of a series of reactions, as fermentation is the overall result of a long series of more elementary events. He will not hesitate to disintegrate the machine and try to dissolve the twitch into its constituent reactions; if he is interested in thermodynamics, he will vary pressure and temperature within wide limits.

The thermodynamic approach will be especially valuable to him for several reasons. First, thermodynamics is not concerned with structure; for its application it is immaterial whether we are dealing with a muscle or a frankfurter. It allows us to start working before the details of structure are clarified. Secondly, thermodynamics allows us to correlate changes observed in muscle with the big driving forces of Nature, such as heat and entropy, potential and kinetic energy. While myothermics and thermometer, owing to their very nature, can only show overall heat changes, thermodynamics may give us information about the single reactions and may even reveal simultaneous opposite heat changes compensating each other.

Thermodynamics is a system of logic which established relations between chemical events, different forms of energy and entropy. It is limited to systems in which there *are* such relations. With other words: thermodynamics deals only with reversible actions, and our

main problem is whether, in muscle, there are such relations, whether muscular activity is built up of reversible reactions, and whether thermodynamics can be applied. The fact that muscular activity entails irreversible decomposition of foodstuffs does not prove that this function is not built up of a series of equilibrium reactions.

Our problem is not whether "muscular contraction" is reversible, but whether from the series of reactions we can bring under observation single equilibrium reactions to which we can apply thermodynamics. There is only one way to find out whether thermodynamics can be applied—to try.

The two main thermodynamic factors are pressure and temperature. Accordingly, we can start out by varying one of these two and observing how the muscle behaves. From our results we can try to extract reactions which look reversible, make the necessary assumptions, and try to apply thermodynamics, expecting to see, eventually, whether the whole story makes sense. This procedure involves the risk of wasting labor, but since it is our own labor we are risking, there is no reason why we should not take the chance.

2. Remarks on Methods

Muscle does three things: it shortens, develops tension, and does work which we can measure at varied temperatures. If we want to apply thermodynamics to our results we must be careful that all responses we measure are maximal, that is, that the whole mass of contractile matter shall have taken part in their production simultaneously. This excludes the twitch from our methods, for in a twitch contraction and relaxation occur simultaneously, all mixed up. Maximum stimulation can be achieved electrically in intact muscle by applying tetanic stimuli of appropriate duration, frequency, and intensity by means of massive electrodes. Equilibria can also be disturbed throughout the whole mass of contractile matter by freezing and subsequent thawing. In the glycerinated psoas the whole contractile substance can be brought to contraction by placing the muscle in an ATP solution. In this material actin and myosin are permanently associated to actomyosin, and above 15°C actomyosin, in the presence of ATP, is stable in the contracted state only; thus in this material at room temperature or above we have contraction without relaxation.

In measuring shortening or tension there is no difficulty. The only difficulty is with the measurement of maximal working capacity. If we load the muscle with the greatest load it can lift, it will lift it only a small distance, and it could lift a small weight farther. The maximal working ability will thus be the sum of all weights the muscle can

just lift, times the corresponding distance. This work integral can be estimated by decreasing the weight gradually until the muscle is unable to lift any further weight and by noting the shortening all the time. Instead of using weights we can also measure the tension developed. In this case we would have to allow the muscle to shorten gradually, while noting the tension. Such an experiment is represented schematically in Fig. 43, where *AD* is the length of the muscle and *DF* the tension developed by it. If we allow the muscle to shorten gradually and note

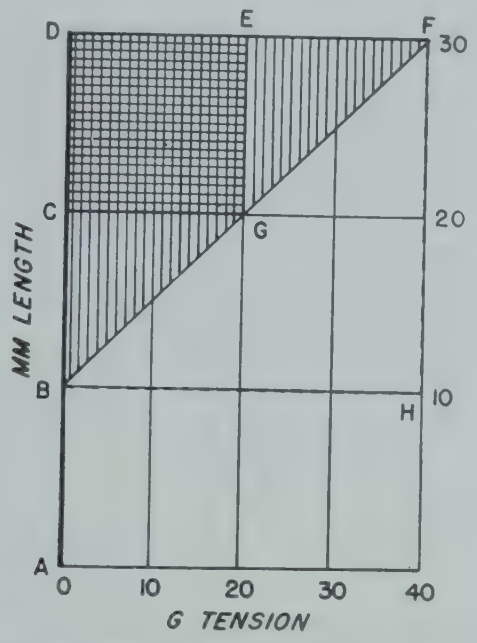


FIG. 43. Schematic representation of the relation of length and tension in muscle. For explanation, see text.

the tension continuously, we obtain the curve *BF*. The sum of all tensions developed at different lengths, the work integral or working capacity, is the hatched area *BDF*.

Such an experiment is very cumbersome, and A. V. Hill devised a shortcut thirty years ago. He estimated the work integral for his material, the frog sartorius, and found that this was one-sixth of the product $l \times t$, the length of the muscle multiplied by the maximal tension. Once this factor was established he had only to measure the length and tension and multiply their product by one-sixth, and he had the maximal working capacity. This method has three advantages: (1) it is simple, (2) it is devoid of assumptions, and (3) the curves obtained are practically identical with the curves of maximal tension.[1] Whether the curve stands for work or tension will depend

[1] The factor varies only slightly with temperature.

only on the units of the ordinate. This also simplifies our job, and we can limit ourselves to the discussion of two curves, the curves of shortening and the curves of working capacity.

We have to refer the working capacity to a certain quantity of muscle. We are free to use any unit, for instance, the working capacity of 1 g or kg of muscle. In the subsequent pages the work will be expressed in calories and will refer to 70,000 g of myosin or the quantity of muscle which contains 70,000 g myosin. This quantity was chosen for various reasons. First, it is one of the basic protein units. Secondly, the P content of myosin suggested the presence of such units. Thirdly, this quantity of myosin can do 4400 cal worth of work at 0°C in the frog sartorius, which is 40 per cent of 11,000. A. V. Hill has shown that at 0°C the sartorius works with 40 per cent efficiency; 70,000 g myosin is thus the quantity of myosin which can transform the 11,000 cal of free energy of ATP into work with 40 per cent efficiency. Fourthly, this is the quantity of myosin which according to the contents of the previous chapter reacts with one actin ovoid, forming the smallest morphological contractile unit.

3. The Behavior of Muscle at Varied Temperatures

If we wish to find out which of the reactions of muscle are expressions of basic regularities, we had best compare a varied material and find out which of the features observed are common. For this purpose the ATP contraction of actomyosin threads was compared with the contraction of intact rat diaphragm muscle excited electrically (Hajdu), with the contraction of intact frog *sartorii* excited likewise (Hajdu and O'Sullivan), with the contraction of rabbit's psoas excited by freezing and subsequent thawing, and finally with the ATP contraction of glycerinated rabbit psoas (Varga, 1950). The results are summed up in Fig. 44. In this figure the results obtained with actomyosin threads are omitted, because no tension could be measured in this material. Its shortening showed the same regularities as that of the other four muscle preparations. Figure 44 contains four sets of curves, corresponding to the four materials. The top curve in every set of curves shows isotonic shortening of unloaded muscle, the middle curve the maximal working capacity (or, which is almost the same. maximal tension). The punctuated parts of the curves are extrapolated. For the time being we will disregard these parts, as well as the lowest curves drawn with a broken line. Temperature was varied (abscissa). All changes in these curves shown with full lines were reversible.

No more heterogeneous material could have been selected. In

spite of this heterogeneity, Fig. 44 shows a striking uniformity of behavior. Shortening and tension depend on the temperature in a singular way, increasing with it at first and later decreasing with its further elevation.

The top curves of isometric shortening of unloaded muscle show that at any given temperature one and the same kind of muscle always shortens to the same extent. Shortening is a function of temperature, suggesting a temperature-dependent equilibrium.

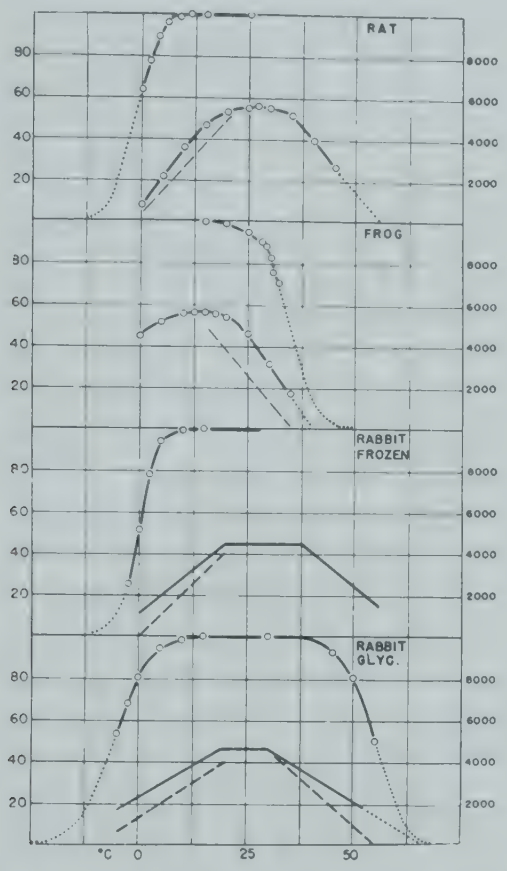


FIG. 44. Behavior of various muscle preparations at varied temperatures. Top, rat diaphragms, excited electrically; second, frog sartorius, excited electrically; third, rabbit psoas excited by freezing and subsequent thawing; bottom, glycerinated rabbit psoas contracting in ATP. Left ordinate, per cent contraction (100% = maximal contraction, 0% = no contraction); right ordinate, maximum working capacity of 70.000 g of myosin (rat, frog) or actomyosin (rabbit) in calories. Top curve in each set is per cent isotonic shortening (unloaded, left ordinate). Punctuated lines, extrapolated; middle curve, maximum working capacity: "$\Delta F'$": broken line, ΔF^0 calculated from shortening. $\Delta F'$ and ΔF^0 refer to right ordinate. Numbers mean calories.

Objections might be raised against this idea of an equilibrium since it belongs to the definition of an equilibrium that can be reached from both sides. The final point, in this case, can be reached from one side only, since voluntary muscle does not relax actively (A. V. Hill). In other words, if we make a muscle contract at a given

temperature, it will not stretch out if we bring it to another temperature, which according to the curve corresponds to a greater final length. This is true but does not exclude thermodynamic reversibility, as can be illustrated by a simple example. If we take a metal rod, place it on the table, heat it, and cool it alternately, it will lengthen and shorten, there being a thermodynamic equilibrium at any point. Thermodynamics is not concerned with structure, therefore we are allowed to make the rod similar to muscle by cutting it up lengthwise into a bundle of thin wires. By doing so we change none of its thermodynamic properties, but it will, nevertheless, now behave differently, similarly to muscle—when cooled it will shorten, but when warmed it will not stretch, the thin wires will fold up instead.

Thus we are still justified in trying to apply thermodynamics. The question is only how? Equations of thermodynamics mostly involve the idea of the relative number of small units, e.g., molecules. Contractile matter is built of small functional units, be it ovoids, globules, or molecules. The problem is only how to find out something about their number. Buchthal and Knappeis (1943) studying physical properties of muscle arrived at the conclusion that the contractile matter is built of small units reacting in an all-or-none fashion. The all-or-none nature of the reaction is a most probable one, for most physical changes in proteins, like denaturation, are as far as we know all-or-none reactions of the first order. We have seen that contraction in actomyosin is very similar to heat denaturation, thus the most reasonable assumption we can make is that the macroscopic shortening of muscle is an integral of the shortening, an all-or-none first order reaction of small units. If this is so, then from the change in length we can simply read the relative number of units which have reacted and of units which have not. Maximal contraction (100 per cent on left ordinate in Fig. 44) means that all units have contracted; no contraction means that 0 per cent of the units have contracted. If at a given temperature the muscle contracts halfway between 0 and 100 per cent, this means a fifty-fifty arrangement, half of the units being contracted, half of them uncontracted. Contraction by three-quarters of the maximum would mean three contracted units for one relaxed one. This relative number of reacting and non-reacting units is nothing but K, the equilibrium constant of the reaction, which depends on temperature and thus can be read from the extent of shortening, provided our assumptions were correct.

The assumptions made were the most probable ones, and there are even common, everyday phenomena which conform to them. Let us take an example, a swimming pool, with steep walls and many people. These people will constantly be going in and out of the water, and there will be a constant relation between people in the water and those out of it. There will thus be an equilibrium reaction, and the relative

number of people in and outside the water will be K, the equilibrium constant. As to our assumptions:

1. The system is composed of single independent units (people).
2. The reaction will be an all-or-none reaction (diving people do not stop in mid-air).
3. It will be a first order reaction, the number of diving people being a direct function of the number of dry people.
4. K will depend on temperature.

Different thermodynamic rules and equations apply to the temperature dependence of the K of an equilibrium reaction. One such rule is that the log of K, if plotted against $1/T$ (T = absolute temperature), should be a straight line if the reaction is a simple and not a composite one.

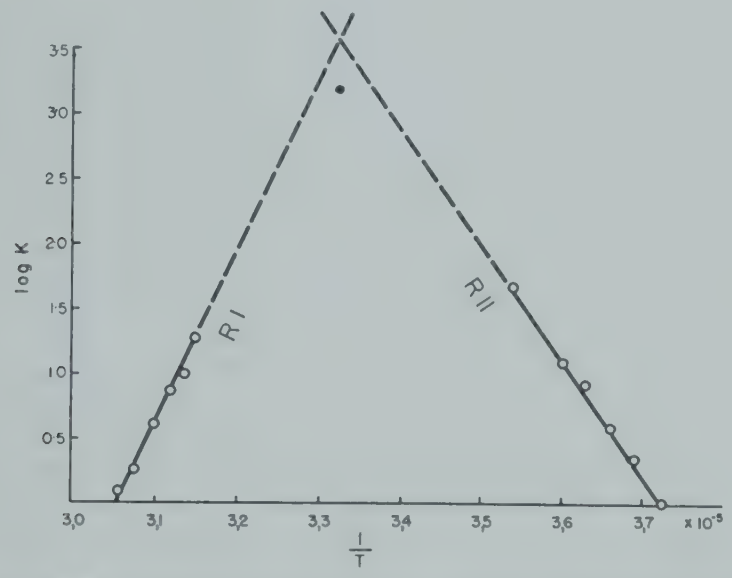


FIG. 45. Log K calculated from shortening of unloaded glycerinated rabbit psoas, contracting in ATP, plotted against reciprocal value of temperature. Broken line, extrapolated linearly.

If we try to apply this rule to our muscle, e.g., the glycerinated psoas, read K from the final length reached in isotonic contraction at various temperatures and plot the log K's against $1/T$, we obtain a curve like that shown in Fig. 45. The broken line is extrapolated and corresponds to the highest degrees of contraction where the K cannot be ascertained with sufficient accuracy. The result is not one but two straight lines indicating that we are dealing with a composite reaction composed of two single reactions, let us say R_1 and R_2. Similar curves are obtained with the three other materials. The actual shortening is governed at lower temperatures by R_1, which at these temperatures has the lower K. At high temperatures the behavior is governed by

R_2, which has the lower K's at these temperatures. This suggests that the shortening observed is the macroscopic result of two reactions coupled in a peculiar fashion. Contraction is the result of their joint action. Its bottleneck (limiting factor) at any given temperature is that one of the two reactions which has the lower K. If R_1 (Fig. 45) is symbolized by $A \rightleftharpoons B$, then its K_1 is $[B]/[A]$. If R_2 is symbolized by $C \rightleftharpoons D$ then K_2 D/C, and the contracted matter is BD. At a low temperature K_1 is small, yields little B, and governs the behavior, while at a high temperature K_2 is small, yields little D, and is dominant. So, although contraction itself is a complex coupled reaction, at low temperatures it will behave as if it were one simple reaction, R_1, its kinetics being governed by this reaction. At higher temperatures the same will be true of R_2.

A straight line is statistically most improbable, because it is just one of an infinite number of possibilities, and it is highly improbable that we should have obtained one without some underlying thermodynamic regularity. If, however, this straightness does not carry too much weight, this is because the log makes the curve rather insensitive and tends to straighten it out.

What we have done until now has been to measure shortening and to derive, by means of certain assumptions, numbers which we believed to be K's, the equilibrium constants of the reactions. We then started to study whether these numbers conformed to the requirements of K's, and our first demand was that their log, plotted against $1/T$, should give a straight line, and this we found actually to be the case. The question is what other critieria could we find to ascertain whether or not K's are really K's. According to Van't Hoff's isochore

$$\Delta F^\circ = RT \ln K,$$

the natural log of the K of an equilibrium reaction, multiplied by RT should give ΔF°, the free energy change of the reaction. This opens the hope of checking our K's along an entirely independent route, by measuring the working capacity, for the free energy lost by a system is in close relation to the external work it can do. The trouble is only with the little "o" superior to the F, which means that the equation holds good only under standard conditions, that is, if activity is unity and there is no change in concentrations. If we want to calculate the actual work the system can do from ΔF°, we must correct for activity as well as for the changes in concentrations.

There are three reasons for believing that the correction for activity in the present case is insignificant. (1) The molar concentration of myosin in muscle is very low, bringing concentration and activity

close together. (2) It is probable that the activity coefficient of relaxed and contracted protein is identical, and thus the same correction should be applied to the numerator and denominator of K, which cancels out the correction. (3) Even if activities are very different from concentration, and are different for the contracted and relaxed matter, this will not trouble us, since we are not seeking absolute numerical values but merely want to compare the K's obtained from a piece of muscle with the working capacity of the very same material.

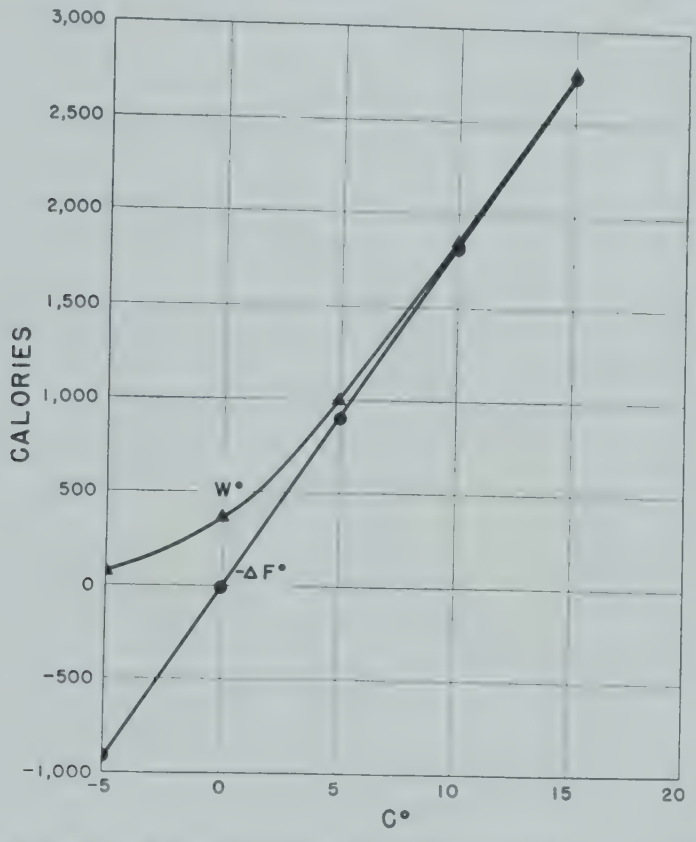


FIG. 46. $\Delta F°$ and $W°$, the calculated maximal working capacity of the same contractile matter. $W°$ corresponds to $\Delta F'$ in text. (Gergely, 1950.)

This will cause the activity to distort K and ΔF equally so that the muscle makes its own corrections.

Gergely (1950) showed that the other correction, the correction for change in concentration, can be taken care of by calculating with $1 + K$ instead of K. While $\Delta F° = -RT \ln K$, the actual work done by the system $\Delta F'$ will be: $\Delta F' = -RT \ln (1 + K)$. This $+1$ means that $\Delta F'$ can never become negative (there being no such thing as negative work). Where K is large, at higher degrees of shortening, this $+1$ will not make much difference and can be ignored (Fig. 46). The 1 begins to matter where K itself approaches unity and thus

ΔF° approaches 0 because of the logarithmic nature of the equation, log 1 being 0. These low degrees of contraction are outside the region in which tension was measured, thus the whole correction does not influence our results in Fig. 44, and we can identify the ΔF° calculated from K and the actual working capacity, $\Delta F'$, which we measured experimentally. We cannot expect complete numeric agreement for our experimental difficulties and consequently our sources of error are considerable. Moreover the material from which K was derived, that is, the muscle shortening in unloaded condition, and the material on which $\Delta F'$ was measured, are not exactly identical, the contractile matter being under tension in the latter but not in the first case. Schauenstein has shown that tended actomyosin has an ultraviolet spectrum which is different from the spectrum of the untended one. Schauenstein and Treiber have also shown that the IP's are different in the two conditions, so no exact numerical agreement can be expected. What we are interested in here in the first place is not the agreement of numerical values but the type of curves obtained and their trends.

The ΔF°'s, calculated from our K's are reproduced in Fig. 44 by broken lines. They are composed of two straight parts, an ascending and a descending one. That these curves are straight is not surprising: if the plot log K versus $1/T$ is straight, the plot RT ln K versus T must also be straight. What is surprising is that the experimental curves of working capacity (the four middle curves which refer to the right-hand side ordinate, on which numbers are calories) are also straight, composed of two straight lines with a smoothed-out transition. Not only are they straight but they also have approximately the same slope as the ΔF° with which they almost overlap. They are really straight, as shown by the more detailed curve in Fig. 47. Such a straight line would be highly improbable if it were not the expression of a deeper thermodynamic regularity. For us this close relation of the two curves, the ΔF° derived from isotonic shortening and the $\Delta F'$ measured in isometric contraction, are important because they were obtained along entirely independent routes by means of different techniques, leaving little doubt that the path we have taken is correct.

The ΔF° refers to the mole. Since ΔF° and $\Delta F'$ are closely related and have almost identical slopes, this proves at the same time that 70,000 g of myosin is actually the weight of the mole, that is, the weight of that part of the myosin molecule which acts as an independent unit, an autone.[2]

[2] If the weight of the unit is 35,000 g, the $\Delta F'$ should be twice as steep as ΔF°'s; with 140,000 g, it should be twice as flat.

Varga used 70,000 g actomyosin as the unit instead of 70,000 g myosin. Since actomyosin contains but a one-fourth of actin, the difference is not very considerable.

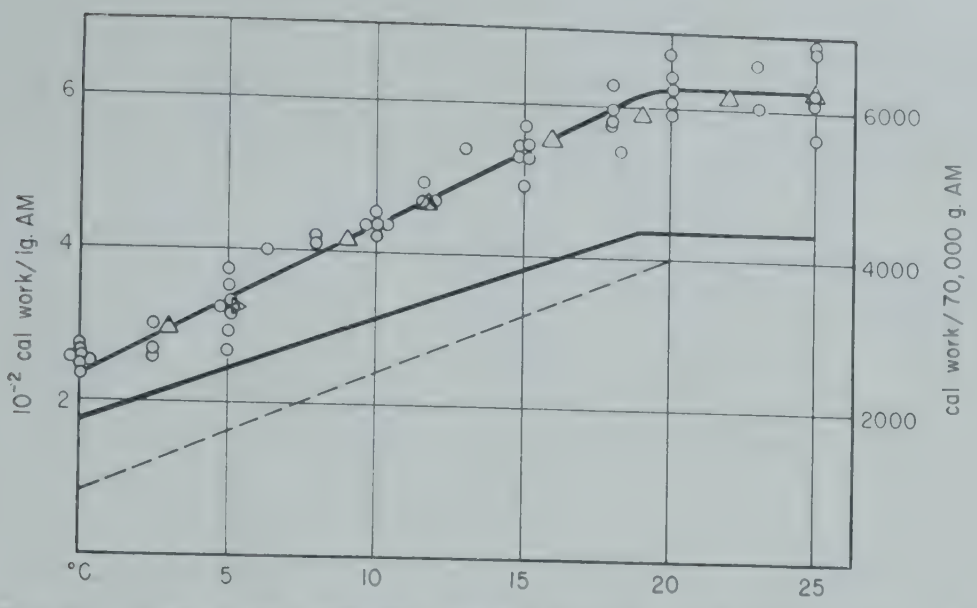


FIG. 47. Upper curve, maximal working capacity of glycerinated psoas fibers (left ordinate) contracting in ATP; middle curve, average value of same, calculated for 70,000 g actomyosin present (refers to right ordinate); broken line, ΔF° calculated from isotonic shortening (refers to right ordinate). (Varga, 1950.)

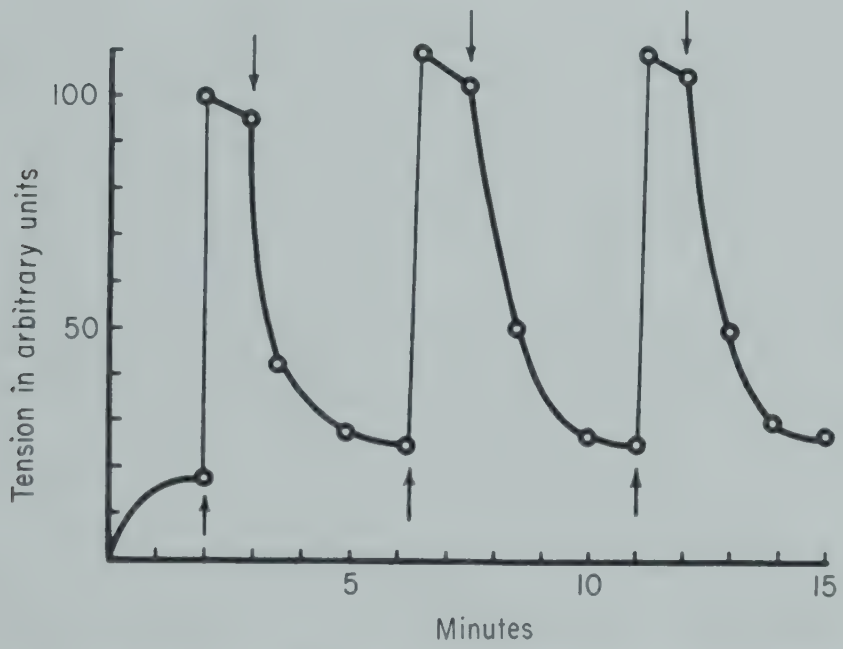


FIG. 48. Tension developed by a glycerinated muscle fiber at varied temperatures in the presence of ATP. At beginning temperature = 0°C. At points indicated by lower arrows, fluid was exchanged for ATP solution of 15°C. at points shown by upper arrows for ATP of 0°C. (Varga, 1950.)

Naturally the correctness of all we have done so far depends on whether the development of tension *is* a reversible equilibrium reaction. If it is, if the curves of tension (working capacity) are the expressions of an equilibrium reaction, then we should be able to change the tension developed by the muscle by merely varying the temperature.

A flawless demonstration of this reversibility has been given by Varga (1950) on the glycerinated psoas contracting in ATP. One of his experiments is reproduced in Fig. 48. A glycerinated psoas fiber connected to the isometric lever was immersed into Ringer containing 0.2% ATP of 0°C. At the first lower arrow, the fluid was exchanged for an identical fluid of 15°C. The tension increased instantaneously, overshooting somewhat, and settled in a few seconds to a final value. Then (first upper arrow) the muscle was put back into the first colder fluid. There was an immediate drop, which approached a final minimum asymptotically. Then the experiment was repeated with the same result, the tension developed at 15°C being about four times higher than at 0°C, as demanded by the $\Delta F'$ curves. It is hardly possible to give a more perfect demonstration of thermodynamic reversibility. If there is no ATP, naturally there is no contraction, no tension, but there is also no relaxation. Figure 49 shows what happens if we repeat this same experiment using ATP-free Ringer when we revert from a high to a low temperature (single arrow). The tension begins to drop, but just before relaxation has reached higher degrees, the ATP is washed out and the whole system freezes up to "melt" again when ATP is added (double arrow).

4. A Little Thermodynamics

Since we have found $\Delta F°$ and $\Delta F'$ to be very similar both theoretically and experimentally, we can amalgamate them simply as ΔF. We will thus simply talk about ΔF and use the numeric values of our earlier $\Delta F°$.

ΔF, according to classical thermodynamics, is equal to $\Delta H + T\Delta S$, where H is heat[3] and S stands for entropy. This equation corresponds to the general form $a = b + cd$ which is a straight line if H is a fixed magnitude which does not change with temperature. The slope of the curve, in this case, due to the entropy contribution, $T\Delta S$. is the entropy slope.

The heat of the reaction can be calculated in different ways. It can be derived from K,[4] the ΔF slope,[5] or the slope of the ln K —

[3] *H*, more exactly, stands for enthalpy. However, since there is no change in pressure it can be identified with heat.

$1/T$ plot. All methods yield identical values, indicating a major endothermic heat change in R_1 and a somewhat greater exothermic heat change in R_2, both between 50,000 and 80,000 cal, this being the range in which protein denaturation is mostly found. In R_1 there is an increase in entropy by almost 200 units, and the system does work be-

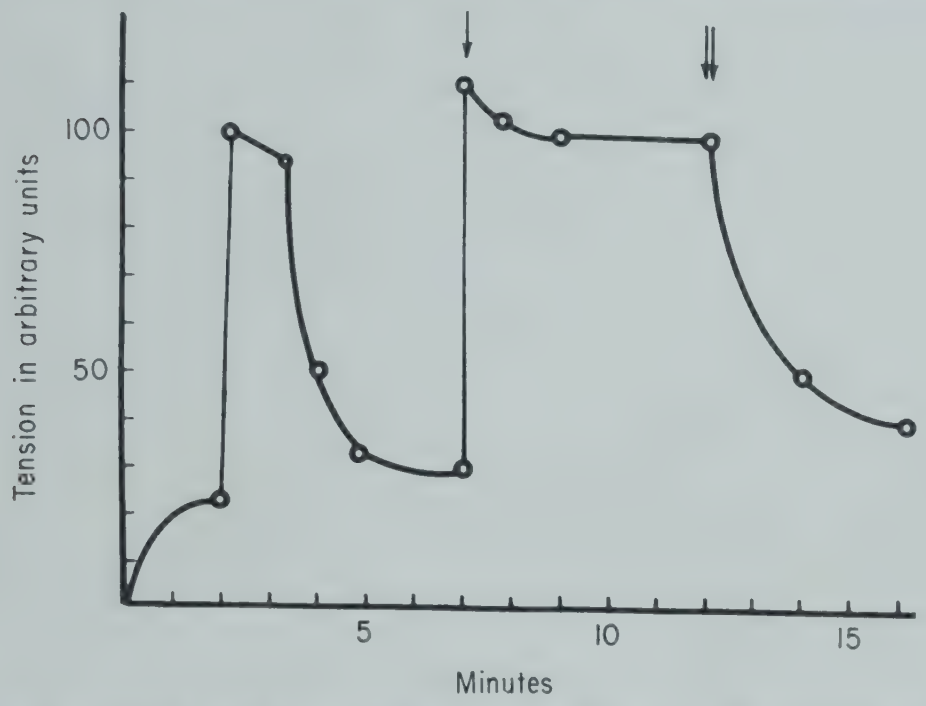


FIG. 49. Same as Fig. 45. At double arrow, warm ATP was replaced by ATP-free Ringer of O°C. (Varga, 1950.)

cause the entropy change overshoots the heat uptake, whereas the reverse is true for R_2.

According to these results contraction is thus brought about by two reactions coupled in a peculiar fashion, each of which has its own ΔF.

The whole situation is summed up schematically in Fig. 50. The 0 line in the middle, used as abscissa, corresponds to $\Delta F^\circ = 0$. The Kelvin scale (absolute temperature) is used as its units. The two ΔF slopes, that of R_1 and R_2 are linearly extrapolated. The top of the little quadrangle corresponds to $\Delta F^\circ = 11{,}000$ cal. Up to this level the energy expense can be covered by the free energy of the P—O—P link of ATP. If $\Delta H = \Delta F + T\Delta S$ and $\Delta F = 0$, then

[4] $\dfrac{d \ln K}{dT} = \dfrac{W}{RT^2}$.

[5] $\dfrac{\Delta F_2}{\Delta T_2} - \dfrac{\Delta F_1}{\Delta T_1} = \dfrac{H(T_2 - T_1)}{T_1 T_2}$.

$\Delta H = T\Delta S$. This is the situation for R_1 at the lower left corner of the little quadrangle. Up to this point the entropy contribution has been compensated by the endothermic heat change, which corresponds to the triangle enclosed by the 0 on the ordinate, angle α and the left lower corner of the quadrangle. If above this temperature the reac-

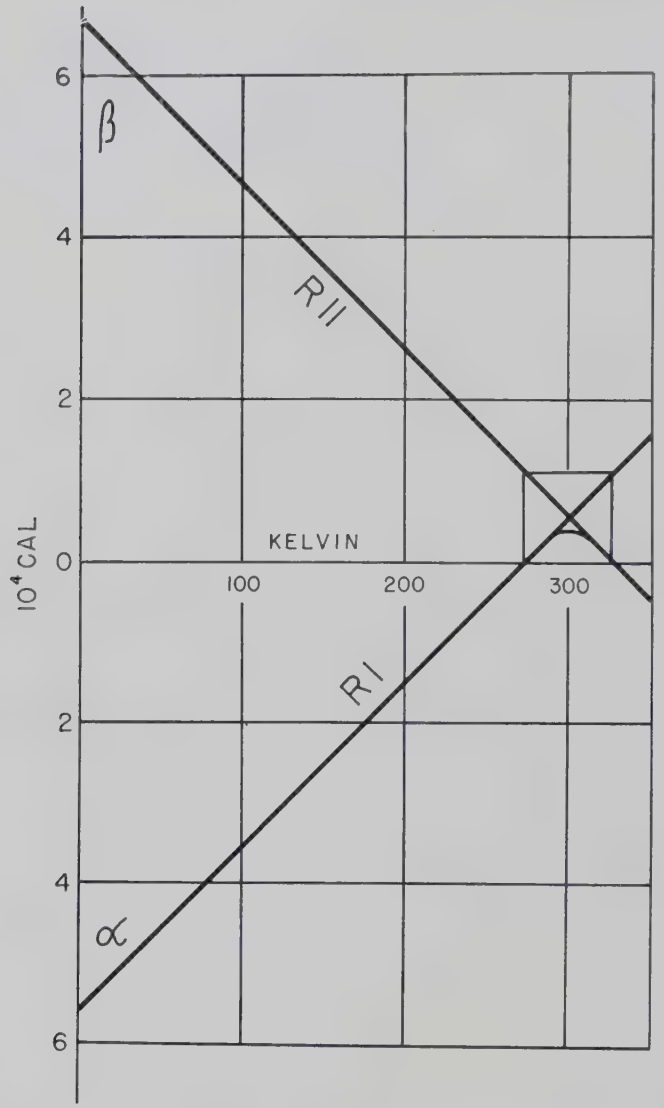


FIG. 50. ΔF curves on the Kelvin scale. Quadrangle is area of reversible contraction with ΔF not exceeding 11,000 cal. See text.

tion can do increasing amounts of work, it is because the increase in entropy progresses without being compensated by the heat uptake any farther. The situation is analogous for R_2 at the lower right corner of the quadrangle. Here the entropy and heat change are opposite, the reaction is exothermic, and the entropy decreases with increasing temperature. The heat change corresponds to the triangle

enclosed by the lower right corner of the quadrangle, the 0 point on the ordinate and angle β. Here heat changes compensate the entropy contribution, $\Delta F = 0$ and $\Delta H = T\Delta S$. If the reaction does more and more work as the temperature decreases, this is because the negative entropy contribution compensates the heat change less and less.

R_1 thus contributes more and more to the work as the temperature increases, R_2 contributes less and less. As the two reactions are coupled, the theoretical maximum of work done by the system lies at the crossing point of the two curves, at $\Delta F = 5500$ cal. Since this is paid for by the 11,000 cal liberated in splitting the P—O—P link of ATP, the theoretical limit of efficiency is set at 50 per cent. This, naturally, will never be reached, the actual curve being smoothed out, as shown by the curved line below the crossing point in Fig. 50.

What is rather unexpected in this whole picture is the magnitude of heat and entropy changes. They are of a different order of magnitude from the actual work done by the muscle—they are higher by a whole dimension. While the maximum work the muscle can do is 5500 cal, the heat change is ten times greater and so is the entropy contribution with the corresponding change in thermokinetic energy. Great forces are at play, compensating one another, and the actual work of muscle is their relatively small over-all result.

This whole story is so unexpected that one would feel inclined to lose faith and believe that one has been led astray altogether if this picture did not completely overlap with results arrived at by chemical study. While thermodynamics shows two reactions coupled in a peculiar way to perform work, chemical analysis brought to light two proteins, actin and myosin, coupled in a peculiar fashion to form a complex, only the complex being contractile and capable of doing work. We were also led to believe by these chemical studies that the myosin particle was kept straight by its internal energy, its system of charges, which were neutralized in contraction, and that the actual work at room temperature was done by the folding, that is, by the thermokinetic energy, the entropy contribution. Thus the chemical studies also suggested great energy and entropy changes, thus overlapping the thermodynamic picture.

One final remark should be made about extrapolation in Fig. 44. The $\Delta F'$ curves were extrapolated simply by lengthening the experimental curve. This is incorrect in so far as the correction for concentration would make itself felt just in this region and the curve would reach the abscissa asymptotically.

If such linear extrapolation was thus not quite correct in the case of $\Delta F'$, it would have been entirely wrong in the case of the curves of isotonic shortening (top curves). Since in the logarithmic scale the curves are straight, the correct way to extrapolate is to prolong the straight logarithmic line and count back. This gives an

S-shaped curve with its turning point at 50 per cent contraction, where $K = 1$ and $\log K = 0$.

Attempts were also made earlier by Varga (1948) to calculate activation energies out of the rate of isotonic contraction. As the rates were limited by secondary reactions and diffusion, the results cannot be accepted.

5. Two Controls

The Webers and the Wiegand-Snyder Equation. The whole story developed in this chapter is so extraordinary that one cannot feel at ease until it has been corroborated by independent evidence, even if the concept is supported by chemical experience. Such evidence was produced by A. and H. H. Weber who approached thermodynamics along an independent route involving but one single assumption: that the reactions are thermodynamically reversible. From the second law of thermodynamics Wiegand and Snyder derived an equation for elastic bodies, such as rubber,[6] which reads:

$$P = \left(\frac{\partial E}{\partial l}\right)_T + T\left(\frac{\partial P}{\partial T}\right)_l$$

which tells us that P, the elastic force, depends on two factors, the internal energy and the entropy, that is potential and kinetic energy. The first, corresponding to the first member of the equation, can be measured by measuring the tension at varied length and constant temperature, the second, represented by the second member, by measuring the tension at varied temperatures while keeping the length constant.

The Webers applied this equation to the ATP contraction of the glycerinated psoas, where P of the equation corresponds to the tension developed. They worked at room temperature and could thus only measure the changes in energy corresponding to R_1, the reaction with the ascending ΔF slope. Their results show that "in its ATP contraction the extracted muscle fiber works as an entropy machine." According to their calculations, heat must be taken up from the surroundings, increasing the inner energy. This change is slightly overcompensated by the entropy change, the difference enabling the muscle to do work. The Webers find the entropy term for a muscle 1 cm in length and 1 cm^2 in cross section to be 0.1 cal and the mechanical work 0.01–0.02,

[6] If a muscle is made to contract isometrically with its ends fixed, it develops tension. If the ends are released, it shortens. This shortened muscle can be stretched again elastically to its original length and will show approximately the same tension as it showed before we allowed it to shorten, its thermodynamic potential being restored. The isometrically contracting muscle with its tension can thus be looked upon as a shortened muscle stretched elastically to its original length, and thus the equation derived by Wiegand and Snyder for elastic changes of state can be applied.

which, calculated for 70,000 g myosin, means about 5000–10,000 cal of work and 50,000 cal of heat adsorbed, an almost complete agreement with our results.

A related analysis of Varga's results and other *lt* diagrams was made by Laki and Gergely, who arrive at similar results, thus corroborating the results of our thermodynamics (in the development of which Laki himself played a leading role) from an entirely different approach.

Heat and Cold Contracture. Within the quadrangle in Fig. 50 the ΔF's, or their sum, do not exceed 11,000 cal which means that the muscle will be able to pay for the expense by means of the energy of

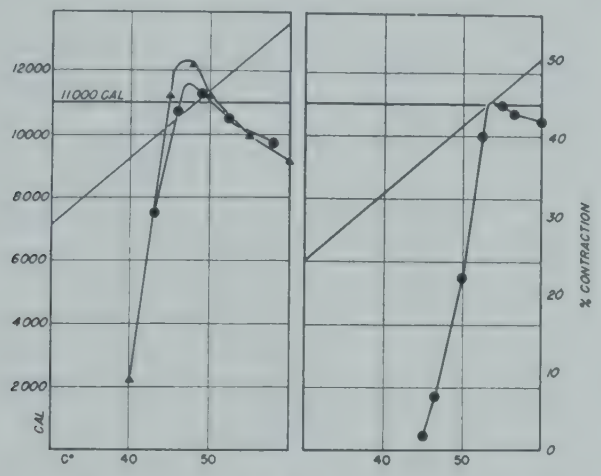


FIG. 51. Left: heat contracture of frog *sartorius*. Right: same for *musculus gracilis* of the rabbit (the author, 1949). Curves relate to right ordinate. Straight sloping lines are extrapolated $\Delta F°$ curves for same material and relate to left ordinate.

the P—O—P link of ATP and relax. Not so if any of the ΔF curves transgresses the limit of 11,000 and leaves the quadrangle. In this case the muscle can be expected to be unable to put the energy balance right and relax, and go into contracture.

The results of experiments on these problems (1949) at the higher temperatures are reproduced in Fig. 51. The sloping straight lines are the $\Delta F°$ curves, while the points mark contraction. As the figure shows, the muscle goes into maximal contracture at the temperature at which the ΔF curve cuts the 11,000-cal level. In the frog, in view of its poikilotherm nature and the different position of the ΔF curve, this happens at a lower temperature. The decrease in shortening at the highest temperatures is due to the incipient heat denaturation.

If our theories are correct, we must expect the muscle also to go into contracture at low temperatures at which the ΔF curve of R_2 cuts the 11,000-cal level. It has been known for a long time that the cooling of muscle may induce contracture. If for instance the muscle is

frozen in liquid air, it becomes frozen in the contracted state, showing that the actual freezing occurs after the temperature, as such, has acted on muscle, bringing it to the contracted state.

Until the present time this cold contracture was unexplained. The ΔF° curve of R_2 affords an adequate explanation. In the mammal the ΔF° cuts the 11,000-cal level at about 0°C, while in the frog the ΔF curve reaches the 11,000-cal level about 10° lower. Hajdu (1950) showed that mammalian muscle actually begins to go into contracture below 0°C whereas the frog muscle does the same at a temperature 10° lower. Since changes are slower at these low temperatures, the effects are not so striking and sudden as on the other side of the quadrangle, but the result leaves no doubt about these relations, which fully bear out our expectations.

6. Physiological Relations

The Limulus. One may wonder why Nature should invoke such violent energy changes in muscular contraction and make the actual work merely the relatively small over-all result. The author can offer only his own explanation which may appeal to the mechanically minded reader. Our body muscles must work fast and find their new equilibrium in contraction quickly. If great forces are at play, this equilibrium position will be found more quickly than if they are weak ones. Let us imagine two spiral springs pitched against each other with a plate between. If this plate is moved out of its equilibrium and released, it will find its equilibrium position again. It will do so very quickly if the springs are strong and more slowly if the springs are weak. The force by which the plate will be pushed into its equilibrium position depends only on the difference in the forces acting on it from both sides and may be the same in the case of two weak and in the case of two strong springs, if only the difference is the same in both cases. The speed, by which the plate will reach its equilibrium position, however, will be slower in the latter case.

Sarkar and Varga (unpublished) tested this concept for its applicability in *Limulus.* The limulus, or horseshoe crab, is a prehistoric animal which forgot to die out. It is an arthropod, a huge insect. Though it has beautiful and strong cross-striated muscles with long straight fibers, its motions are very slow. Its muscles can develop the same force or do the same work as those of the rabbit, but very slowly. We could thus expect to find the values of ΔF the same as in other animals, but the energy changes, which produce this ΔF as their over-all result, relatively small. Smaller energy changes must express

themselves in a flat entropy slope. The upper curve in Fig. 52 shows the curve of tension, developed by the *Limulus* muscle as compared to the rabbit psoas. The lower curve shows the working capacity which has the same maximal numeric values as in other animals, which maximum, however, is reached with a very flat slope.[7]

Tensile Strength and Contraction. We were impressed by the great similarity in the curves obtained with different materials (Fig. 44), but there is one disturbing dissimilarity between the two upper

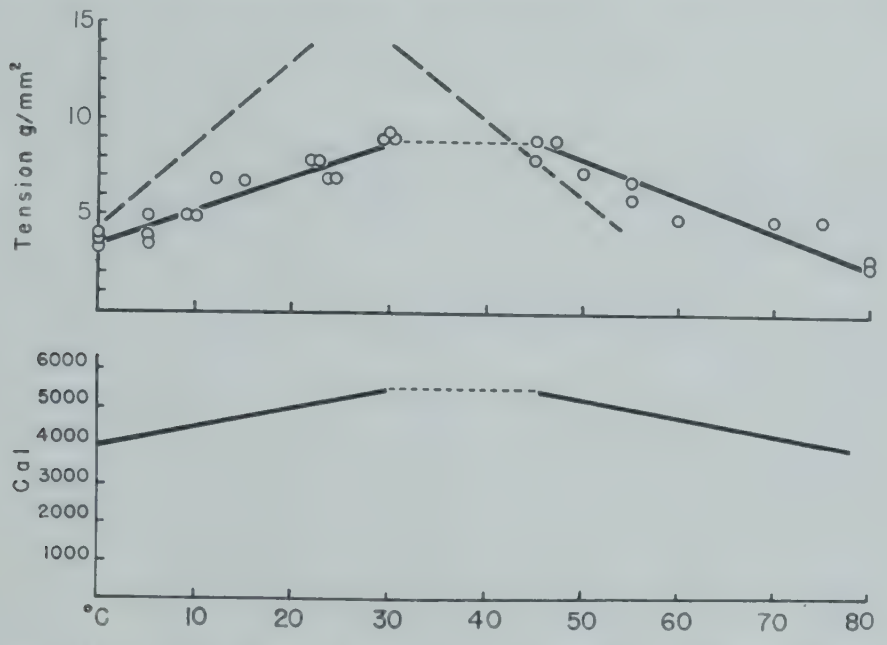


FIG. 52. Upper curve, tension developed by the frozen *Limulus* muscle on thawing; broken line, slopes of glycerinated rabbit psoas, contracting in ATP; lower curve, maximal working capacity calculated from upper curve with Hill factor ¼.

and two lower sets. While in the former the two slopes of the $\Delta F'$ curve pass from one to the other smoothly, in the two lower sets the transition takes place with sudden breaks. I will give only the end of a long story here. The tensile strength of the contractile matter in muscle exceeds only slightly the tension which the muscle can develop, the maximum of which is strictly limited by the crossing of the two ΔF° curves. If the tensile strength is in any way slightly damaged, or is damaged more than the contractility, the muscle must tear. This occurs in freezing and subsequent thawing as well as in glycerol treatment. Thus with this material, at temperatures at which maximal tension is developed, the muscle breaks, as shown in Fig. 53, the microscopic picture (Matoltsy, 1950) of a glycerinated psoas fiber con-

[7] The Hill factor could not be ascertained. The factor ¼ was used, as in the frozen frog muscle.

tracting in ATP at varied temperatures. This picture explains without words why the top of the ΔF curve is cut off, as it were, and why maximal tension cannot be maintained with this material, its drop being irreversible, whereas in the intact muscle changes are reversible throughout and transitions are smooth.

It is most important for the safety of the muscle that the tension it develops shall have a strictly regulated limit, exceeded by the tensile strength. Accordingly to our thermodynamics, the tension developed is strictly limited, so the tensile strength must exceed this only slightly, with the limit well set. That this limit is really set by thermodynamics and is only slightly exceeded by the tensile

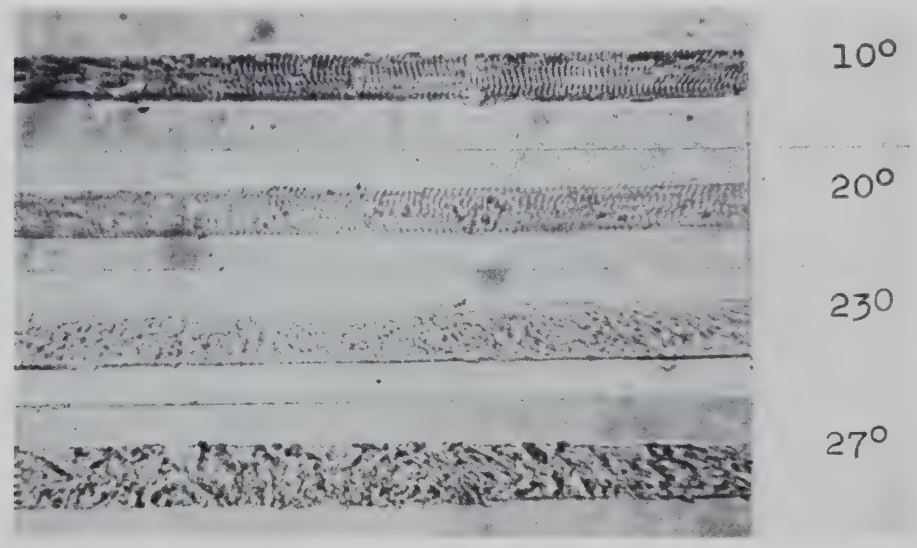


FIG. 53. Microscopic picture of isolated glycerinated psoas fibers contracting isometrically in ATP at varied temperatures. Above 20°C the structure breaks up.

strength had been demonstrated by Varga (1950), who showed that if glycerinated muscle is made to contract at different lengths, it develops different tensions, but these tensions always reach maximum at the same temperature. If the glycerinated muscle is made to develop maximal tension at rest length, maximal tension is reached instantaneously. Nevertheless it takes some time for the muscle to tear, showing that maximal tension and tensile strength are not very different. If the muscle is made to contract from its somewhat shorter equilibrium length, the maximal tension developed will be somewhat smaller and the muscle will not tear at all.

Adaptation, Regulation, Chloroform, Histamine, and Guanidine. As Fig. 44 showed the curves of the frog are very similar to those of mammals, with one difference: they are shifted on the temperature axis to the left, toward the lower T by about 10–12 degrees. This

seems natural, since the muscles of the frog have to work at a lower average temperature. If the frog muscle had the same ΔF as the mammal, it would not be able to move at 0°C at all. As emphasized by A. V. Hill, the frog muscle works perfectly well at 0°C, as is easy to show—provided the stimulus is adequate.

This difference in the frog and mammal poses a most fascinating problem, that of the mechanism of adaptation to temperature. This may have two different underlying mechanisms. Possibly the actomyosin is different in frog and mammal. It may also be that they are similar but their reactions are modified by regulatory substances attached. If a substance becomes attached to actomyosin closely enough, then the two together form a new system and may have new ΔH's, new $T\Delta S$'s, and new ΔF's.

It seems that Nature achieves this adaptation to lower temperature in the frog not by making the actomyosin different, but by attaching to it regulatory substances. This solution of the problem seems to be the more advantageous. A different actomyosin would set the curves once and for all, while the frog has to adapt itself to a whole scale of temperatures as the season changes, which can be done only by regulatory substances. The differences in the reactions of "winter" and "summer" frogs is well known to all physiologists, though an explanation of this difference has hitherto been lacking.

All these differences played a considerable role in the early history of the development of the thermodynamic treatment of muscle and was responsible, to a great extent, for the controversy which accompanied this development. Varga did his first experiments on washed frog muscle and frog actomyosin and found the ΔF° curve obtained very similar to that of the rabbit. The ΔF° was very small at 0°C giving apparently an adequate explanation for the slowness and weakness of the motions of frog at this low temperature. A. V. Hill pointed out, however, that this weakness and slowness was due only to the failure of excitability and the muscle, if properly excited, developed considerable, if not maximal, tension. Hill, in his remarks following the author's paper at the XVII International Physiological Congress at Oxford in 1947 as well as his subsequent papers in 1949, pointed out that all three arguments on which the author's thermodynamics were based were in flagrant contradiction of well-established experimental facts. He pointed out that:

1. The frog muscle developed considerable tension at 0°C while Varga's ΔF curve allowed practically none.

2. The intact frog muscle had no temperature slope at all, the tension developed being, if anything, even higher at 0°C than at 25°C.

3. Contraction of muscle is exothermic, while our thermodynamics indicated a strong endothermic heat change.

A thermodynamic theory which is in contradiction to all classical experimental facts cannot expect an enthusiastic reception. Today all three contradictions are resolved, and if this thermodynamic approach has been developed at all it is owing to a great extent to A. V. Hill's criticism, made in the most sporting scientific spirit.

It was the first objection which kept the author busy in 1947–48, his first year in Woods Hole. It could be shown that washed and unwashed frog muscle behaved differently—intact muscle behaved according to Hill, washed muscle according to Varga, giving different ΔF curves. It was also soon found (1949) that chloroform made the unwashed frog muscle behave similarly to washed frog muscle, the change being reversible. Chloroform at lower temperatures depressed the function, thermodynamically making a rabbit out of the frog. Though this reversible change somewhat increased the author's hope that his thermodynamics was correct, it was not any too impressive: it is no wonder if saturated chloroform vapor, which kills all life, can depress a biological activity.

The study of chloroform was later taken up by Hajdu (1950) who demonstrated the remarkable fact that while saturated chloroform depresses the activity of intact frog muscle at lower temperatures, it greatly increases it at higher temperatures. What chloroform produced was not a *depression* but a *shift* on the T-axis which was reversible, showing that the ΔF curves can be shifted up and down on the T-axis by pharmacological agents. This action of the chloroform also suggested that the regulatory substances which slowly shift the ΔF curve in the winter to lower temperatures in the frog are lipins.

Encouraged by these results, Varga (1950) studied a number of different pharmacologically active substances. Although adrenaline, ephedrine, rhyanodine,[8] caffeine, and carnosine were found to be inactive, histamine and guanidine produced characteristic changes in the ΔF curve, as shown in Fig. 54. Histamine shifted the ΔF curve to the left, to lower temperatures, while guanidine shifted it in the opposite direction, increasing at the same time the tension developed and making the entropy slopes rather steep, thus evidently also increasing the heat changes. These results of Vargo give a beautiful example of applicability of thermodynamics to the analysis of pharmacological reactions, which can thus be correlated with the underlying energy changes.

Hill's third objection was met by the study of the descending

[8] The author is obliged to E. Merck and Co. for the crystalline preparation.

slope of the ΔF curve, which revealed a simultaneous exothermic heat change in excess of the endothermy, leaving as over-all result a slight surplus. This may answer for the relatively small exothermic heat change observed by Hill in his classical studies. Hill's second objection caused the most prolonged headaches. Figure 44 shows why. The ΔF curve in the intact frog is shifted to the left to such an extent that the greatest part of the ascending slope falls below 0°C, at which temperature the muscle freezes, making experimentation impossible. Only the last flattening bit of this slope is left, which very soon goes over into the flat maximum. We thus have only a very narrow tem-

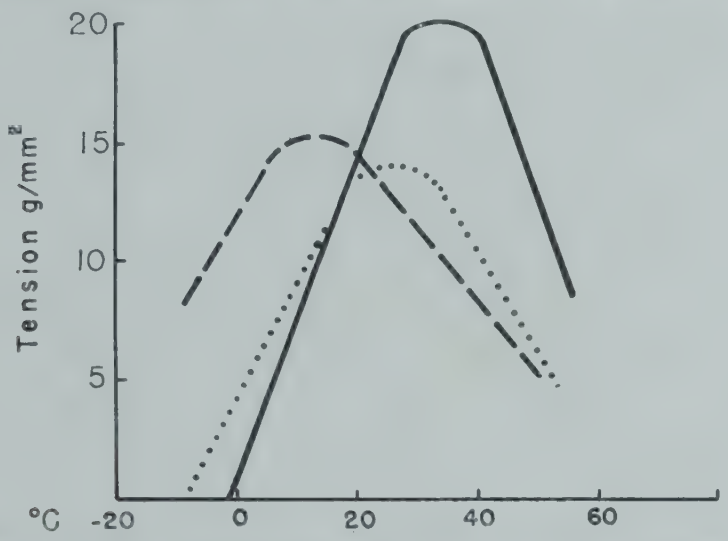


FIG. 54. Punctuated, glycerinated rabbit psoas contracting in ATP; broken line, the same in presence of 0.1% histamine; full line, the same in presence of 0.1% guanidine.

perature range in which the slope could be observed and even in that narrow range the slope is rather flat. So the differences in tension which could be observed with increasing temperature are rather small, while the individual variations between frog and frog are rather great so that the gentle slope cannot be discovered without very special precautions. These precautions were taken by Hajdu and O'Sullivan, who limited individual variation to a minimum by taking the parallel muscles of one and the same animal under identical conditions, or by establishing differences on one and the same muscle, always applying maximal stimuli (which are different at different temperatures). Our own curve in Fig. 44 fully bears out Hill's statement that, if anything, the frog muscle works better at 0°C than at 25°C.[9]

One more point requires discussion in Fig. 44. The author, as any

[9] The author has lately been informed by A. V. Hill that he was also able to convince himself of the ascending slope.

physiologist, would expect the maximum of the ΔF curves to coincide with body temperature. This, as Fig. 44 shows, is not the case. For instance, in the rat diaphragm maximal tension is developed around 27°C, while the body temperature is around 40°C. The body temperature falls to a point on the downward slope and one wonders why this is so. What could be the physiological sense of such an arrangement? The author can offer only his tentative explanation. As we have seen, the tensile strength of muscle is very close to the thermodynamic limit of tension. Nature, in this case, does not show its lavishness, exhibited at other points so often. It seems that the production of tensile strength is a rather difficult problem and that the tensile strength and the maximum of contractile force are dangerously close together. It seems not unreasonable to suppose that Nature wants to reserve this maximal tension for cases of emergency only, when maximal performance is needed. As we have seen, the ΔF curve can be shifted up and down on the T-axis by different substances. If body temperature coincided with the maximum of ΔF, then regulation would not be possible and any shift would bring about only a decrease in tension. With body temperature on the downward slope, regulation of tension is possible both ways by a shift on the T-axis. We have seen earlier that the ATP-ase activity of myosin is regulated by a shift on the pH-axis. It seems not unlikely that Nature achieves regulations by making one of its great parameters the axis of a coordinate system and then shifts curves along this axis. In the present case the tension developed would be regulated by a shift on the T-axis.

One of the most classical phenomena in physiology is heat narcosis, studied by Claude Bernard. Frogs fall asleep around 40°C. The frog's ΔF° curve in Fig. 44 shows that this is the temperature around which the muscle also fails to develop tension, ΔF° becomes 0, showing that heat narcosis is equally a reaction of brain and muscle, is a general phenomenon, and that the behavior of frog muscle at higher temperatures is merely one of its instances. This suggests that the thermodynamic regularitites found in muscle are expressions of the basic, general rules of living matter.

Different organisms, adapted to different temperatures, must have their individual ΔF curves, and a change in temperature may favor one while it disfavors the other. This may explain why our body raises its temperature as one of the means of fighting invading microorganisms. The observation of Pasteur may also be recalled that chickens can be infected by anthrax only when their temperature is lowered, suggesting that the thermodynamic analysis of biological phenomena may open the door for the understanding of even such mysterious and abtruse phenomena as infection and immunity.

Energetics and Mechanics

The terminal P—O—P bond of ATP contains 11,000 cal of free energy (Meyerhof) and is split in contraction. Our problem is: How can a muscle lift weight with the help of this energy, transforming chemical energy into mechanical work?

Should it be found later that before the energy of this bond is used another high-energy bond is first established on its account, this would not answer our question, but merely shift the problem. So this possibility will simply be disregarded in the remainder of this book. Should it be found later that such a transphosphorylation or any similar transfer of the bond energy takes place, our conclusions would merely demand slight rewording.

Some gunpowder has been wasted in fighting out whether there is a pre- or post-energetization in muscle. Do we release chemical energy to enable the muscle to do its work, or does the contractile matter create work out of its own resources which will have to be refunded afterwards? There is not much sense in this question, for what is post-energetization for one twitch is pre-energetization for the next one. Moreover, the question as it stands suggests the idea that the free energy of the P—O—P link can be liberated and then mailed to the contractile matter for further use. Free energy, "liberated," would turn into heat and be wasted. There can be no "liberation" or "transfer," and the ATP molecule with its energy must be involved more directly in the mechanism of contraction.

Before trying to piece our story together let us review our knowledge of energy and the P—O—P bond.

About the fate and participation of energy in biological processes we know very little indeed. We know no more than we do about the fate of our dollar bills once they have passed over the counter of the bank. We only know that we can get them back, and the only thing which concerns us is the *balance* and not their actual fate. The situation is similar in thermodynamics. The balance sheets have to be taken even more seriously here because, whereas in the bank we

may occasionally overdraw our account, this is most strictly forbidden in thermodynamics (first law). It simply cannot be done. Since this balance is the only thing of which we are sure, we must adjust our mind to thinking in terms of it.

There is just one small limitation: the bank will always charge us something for every transaction, and so there will always be a small loss for us, whatever we do, and we cannot dispose of the whole sum deposited, since not the whole of it is "free." The same is more or less true of energetics where there is always a tendency to dissipate some of the energy as heat, only the rest being "free-energy" (second law). The loss in the case of ATP is very small, therefore we may allow ourselves to be preoccupied solely with the balance of our account which can never become negative. Consequently if actin, myosin, and ATP get together and do work or dissipate energy, they cannot go back to their initial state without balancing the account and releasing an equivalent amount of chemical energy. This is all we really know.

We are not much better off with our knowledge of the "high energy" of the phosphate bond of ATP. "Phosphate bond" needs no explanation, but what does "high energy" mean? The P—O—P bond is often looked upon as a spare box into which we can put energy which we can simply take out later and spend in any way we like. This idea is misleading. The phosphate bond was made by taking out one molecule of water, H—OH, from two molecules of phosphate, H from one, OH from the other, thereby linking the two phosphates together to P—O—P. Eleven thousand calories' worth of work has to be done in taking this water out. All we now know about this energy is that we can get it back by putting the water back, that is, by splitting the phosphate bond again. Naturally, if we do this simply by putting the water in, all our invested energy will inevitably be wasted as heat. We can utilize this bond energy only in certain definite ways.

One way in which we can make use of it is by making the P—O—P take up one special H and OH, the removal of which costs work. If there is a suitable catalyst present, the P—O—P will do this and may pick off any H and OH from another substance, provided that this demands no more then 11,000 cal worth of work, all that P—O—P has. This is illustrated by an example taken from one of Lipmann's (1949) classical papers. If we have a molecule of glycine, benzoic acid, ATP, and a specific catalyst which brings these three together in a specific way, when the ATP will pick off one H and OH from the glycine and benzoic acid, linking the two together to hippuric acid and release its own phosphate, turning itself into ADP. What actu-

ally happened probably was that a single compound was formed for an instant out of the four substances, enzyme, glycine, benzoic acid, and ATP, which then broke up into enzyme, hippuric acid, ADP, and phosphate. What the outside observer sees of all this is that hippuric acid was formed and ATP was "split." Since taking the water out of glycine and benzoic acid costs only 3000 cal and ATP had 11,000, the observer will also notice the development of 8000 cal of heat.

There is also a way in which energy can be taken out of P—O—P without its hydrolysis. Phosphate can form "high-energy-links" not only with phosphate, but also with N or C. The quantity of energy we need to make a C—O—P link depends on the other atomic groups which are linked to the same C. In order to link —O—P to a carbon which has an OH attached to it, we need only 3000 cal. In order to link it to a C which has an =O attached to it, we must invest 11,000 cal, producing a real "high-energy phosphate link," a "~P" as Lipmann denotes it. We can also build such a ~P in the following way: We attach the —O—P to a C which has OH linked to it and make a "low-energy phosphate bond" and then remove the H from the O, shifting it to another C of the same molecule and increasing herewith the energy of the C—O—P link to that of a ~P. Naturally, at the same time we have to make the other parts of the same molecule poorer in energy, so to speak sucking out its energy and pumping it into the ~P established. This is what happens in fermentations in which ~P and the energy-poorer lactic acid are fabricated out of sugar. If this whole process is turned round, then we do the reverse and infuse the energy of the ~P into the rest of the molecule.

To make the story complete, we ought to mention that we can not only release but we can also "transfer" the bond energy together with the phosphate if we have the proper catalysts. Out of a P—O—P we can make a R—C—O—P or some other ~P, without using or releas-

O

ing energy.

Thus a "transfer" of energy from ATP to myosin can be pictured in different ways, but there is one way in which the transfer cannot be done and this is by hydrolyzing the P—O—P, releasing its energy, and mailing it to myosin. The high-energy bond must be directly involved in the mechanism and must take a direct part in the mechanism of contraction, as a piece of the machine, so that its presence shall induce action and its breaking up induce relaxation, at the same time making the "debit" and "credit" side of the energy balance sheet tally again. All these demands are covered by ATP, which induces contraction. ADP does not do so, and therefore we can expect it to be

equally unable to maintain the contracted state while the splitting of the P—O—P satisfies the first law of thermodynamics. The device is most ingenious (as most devices of Nature are): to set the machine going by a substance and to stop it by taking this substance to pieces and hiding between the pieces the energy needed to cover the expenses.

Thermodynamics is one language, chemistry another, and mechanics a third in which we can describe happenings. The three versions of one and the same text should overlap as the three prints of a three-toned picture, which give the correct impression only when together.

By attempting to use the language of chemistry, we can symbolize the presence of a ~P by an exclamation mark, the uncontracted or relaxed state by *R*, and the contracted state by *C*. We could tentatively write:

$$\text{Actomyosin-ATP!}_R \rightleftharpoons \text{Actomyosin-ATP}_C,$$

expressing thereby that in contraction energy has been spent, and if the terminal phosphate of ATP is split off, not the full amount of 11,000 cal of heat will be released any more. Deductions will be made for the energy spent in contraction, which is equivalent to saying that some of this energy has been spent.

This symbolization expresses a rather fascinating possibility. It shows that the complex actomyosin-ATP has two forms, an uncontracted and a contracted one, the two being different in energy content and dimension, with the latter form shorter and wider. When shortening, the system may lift a weight and do work on account of this own energy lost. But if we prevented it from going over into the shortened state by holding the ends fixed, then the reaction could not take place at all, or if we allowed the system to shorten, we could simply revert it to its initial state by stretching it out again, using some external force. This possibility has lately been supported by A. V. Hill's (1950) discovery that excited muscle can actually absorb external work when stretched, storing it as potential or chemical energy. Stretching simply would mean turning the above reaction round making it go in the direction of the lower arrow and making a high-energy bond out of a low-energy one. It is even possible that by stretching we can actually synthetize a ~P. The fact that only "excited muscle" is capable of absorbing and storing work means in our language that only actomyosin can perform this reaction.

This reversibility might open two rather fascinating possibilities. The one is the establishment of a dynamic equilibrium in muscle. The actomyosin units, autones,

are fixed in space and so there can be no collisions between them. This induces a rather serious complication since the application of thermodynamics to chemistry is based on kinetic considerations and presupposes free collisions, bringing the different particles into equilibrium. The stretching of one contracted autone by another could be equivalent in its effect to free collisions.

The other possibility alluded to is the physiological bearing of this reversibility which might open the possibility for muscle to economize energy in a contraction with little shortening. Part of the units could be stretched out by others, limiting the splitting of ATP thus to the smaller number of contracted units.

Shifting to the third language, that of mechanics, the fragmentary nature of our knowledge will prevent making any final statements here also. What the author can do is merely to state what assumptions in his opinion cover the observations best. They are as follows.

Myosin consists of very thin and long particles, kept straight by their charged condition. When actin and myosin-ATP unite in "excitation" to form actomyosin-ATP and the two proteins discharge each other, the linear configuration becomes improbable and the particle, under the influence of heat agitation, folds up. This it will do if allowed to, one of its ends being free. If both ends are fixed, the transition will not take place, but a thermodynamic potential will be established and the trend to spend energy and dissipate the potential in shortening will express itself as tension. If the myosin particle folds up and shortens, the actin ovoids, being built of mobile little globules, ball-bearings, will be able to follow this change in configuration without releasing the myosin. This picture overlaps the picture given by thermodynamics. Thermodynamics showed that there are great changes in distribution of energy and entropy, and contraction is the relatively small overall result of all these changes. According to the above picture, in resting muscle the trend to increase entropy by folding is counterbalanced by potential energy, the highly charged condition of the particle. This charge is depolarized in contraction and the particle folds up, greatly increasing its entropy. As will be remembered, in R_1 the entropy increases by about 200 units, which values is so high that it cannot be explained by an increase in vibrational, rotational, or translational freedom, but only by a change in the configuration of the whole particle. Most of the entropy change we found compensated by heat uptake. The increase in entropy of myosin is accompanied by the increase in entropy of the water which is liberated from its bound condition by the discharge of the protein. While myosin goes over into its more probable configuration, it enforces a less probable one on actin, inducing major changes in the distribution of forces in this protein.

This theory of the mechanism of contraction is an extension of the theory of Wöhlisch, who was the first to make the statistical coiling of elongated particles responsible for muscular contraction. It also covers the observation of Draper and Hodge who saw their period of 400 A shorten in contraction.

Various models have lately been described showing that a folding chain molecule can actually do external work comparable to the work done by muscle. Kuhn, Hargitay, Katchalsky, and Eisenberg produced a highly polymer fibrous polyacid kept straight by the dissociation, that is hydration and mutual repulsion, of its dissociated COOH groups. A decrease in dissociation produced by the addition of acid makes the stretched condition improbable and causes the chains to shorten. Pryor used a natural fibrous high polymer, a tendon with its elastin, which shortens under the influence of mercury potassium iodide. The K_2HgI_2 forms a complex with the free amino groups which were instrumental in keeping the system straight. Kushinsky and Turba recently showed that in actomyosin the NH_2 groups are involved in contraction whereas blocking the COOH's had no effect on this reaction.

There is a fourth language which we can use, that of the U.S. Patent Office which would not hesitate to class the whole system with tip mechanisms, to which most slot machines belong. "Tip mechanism" means that the whole machine is started up by a relatively small change, the nature and magniture of which have nothing to do with the change it elicits. The tip mechanism in muscle is the disturbance of the ionic balance caused by the "wave of excitation" traveling along the membrane. This starts the whole contraction cycle, eventually leading to the splitting of $\sim$P.

The chain of events elicited proceeds all the time spontaneously until the cycle is completed and the system is returned to its initial state. There is, however, no theoretical reason why the series of events could not be stopped at any point. If the cycle is stopped at the point at which the muscle has just developed its tension, the tension could be maintained without considerable expenditure of energy for any length of time. This seems to be the case in clams, which can keep their shells closed practically without using energy or producing action potentials. Crustacean muscle is said to have a double innervation and to need a nervous impulse for contraction as well as for relaxation. Some such mechanism may still be dormant in human muscle and called to action by special conditions of the nervous system, such as hypnosis or schizophrenia.

If the mechanic theory outlined above is correct, it should be possible to produce contraction and tension in muscle even without ATP at high temperatures which

denature and discharge myosin (denatured protein losing its charge easily in presence of ions). The experiments undertaken by Varga along this line tend to show that this is actually possible though tensions are lower and the temperature slope of their curves is flatter. These experiments, in progress now, begin to suggest that the action of ATP consists partly of decreasing the activation energy of denaturation, making reversible denaturation possible at body temperature. It should be remembered that ATP also makes actomyosin elastic similarly to elevated temperature.

The Contraction Cycle

If we had to wade knee deep in theories when correlating chemistry with structure, we will have to wade in theories up to the waist when correlating our *in vitro* experience with reactions *in vivo* since there is incertitude even about the simplest questions of chemistry itself.

1. The State of Rest

Uncertainty begins with actin and its state in resting muscle. Straub (personal communication) is inclined to believe that actin, in resting muscle, is present in its G-form and the first step in excitation is the G-F transformation, which automatically entails actomyosin formation and contraction. The author is unable to share this view. A depolymerization of F- to G-actin would entail such profound disorganization which is hardly compatible with the rate of motion and the precise molecular architecture required. Moreover, if actin were present in its G-form, it should be readily extractable with water, which it is not—we have to apply rather drastic treatment to depolymerize it and bring it into solution. All the EM pictures taken of muscle fixed in fresh condition by formalin indicate the presence of actin ovoids in resting muscle.

The next problem would be to decide whether F-actin, at rest, is associated to myosin or not. All evidence indicates that it is not and that instead of actomyosin resting muscle contains actin and myosin side by side. Muscle contains ATP in considerable (0.2–0.3%) quantity, accessible to myosin; at room temperature and in the presence of ATP, actomyosin would have to be in the contracted state. If resting muscle is not contracted, there is either no actomyosin or no ATP. The efforts made by Rozsa (1946) and later by A. G. Szent-Györgyi (unpublished) to demonstrate that the ATP is present in muscle in an inactive form or is inaccessible to myosin have failed. All experience pointed to the contrary. Thus we are left with the alterna-

tive that resting muscle contains no actomyosin but merely myosin and actin or, if we prefer, contains its actomyosin in the dissociated state. "Dissociation" in this case does not necessarily mean disorganization; it means no more than that the actin and myosin particles are too far part to react chemically. Their mutual distance need be no more than a few angstroms; it depends on the balance of the attractive and repulsive forces which make the particles settle at an equilibrium distance depending on the over-all charge of particles governed by the ionic balance of the medium. The situation is very similar to that found in a crystal of the tobacco mosaic virus, as described by Bernal and Fankuchen who found that "the identical rod-shaped particles of the virus maintained themselves at equilibrium distances which are dependent on the pH and the salt concentration."

It is easy to support this picture of the resting state experimentally. If a freshly isolated frog or rabbit muscle fiber is placed into isotonic salt solution, be it NaCl or KCl, nothing happens, although the system contains actin, myosin, and ATP. If, however, the salt solution is hypotonic, the muscle contracts. Contraction is not instantaneous, since the establishment of the osmotic equilibrium demands some time, but in 1 to 2 minutes the fiber may contract to one-half its original length. Similarly, if the freshly isolated muscle fiber is placed for a few seconds in glycerol with its ends fixed, it contracts vigorously when returned to the isotonic salt solution the balance having been upset. Freezing and subsequent thawing has the same effect. All this shows that actin, myosin, and ATP are all there, ready to act, and do so as soon as the subtle balances of resting muscle are disturbed. In all these instances contraction is elicited by the ATP, which in the resting muscle could not bring about contraction, since no actomyosin was present, but did so as soon as this complex was formed.

2. Excitation

The "wave of excitation" passing along the membrane disturbs the balance of charges, decreasing repulsive forces and equilibrium distances between particles. Below a critical value actin and myosin-ATP join to form actomyosin-ATP. This association is perfectly reversible and by increasing the equilibrium distance by a corresponding change in the ionic balance, we can readily make the complex dissociate again into the component proteins. We have seen earlier that *in vitro* a difference in charge, corresponding to a difference in concentration of 0.02 *M* KCl, is sufficient to drive the two proteins apart or bring them together. So if the ionic balance were restored quickly enough, the actomyosin could be expected to dissociate again without anything fur-

ther happening. Otherwise a series of events is started which go hand in hand with a decrease in free energy, and a full cycle must be described before the system can return to its initial state. In muscle the "wave of excitation" evidently lasts long enough to do this, but is over before the cycle is completed.

Actomyosin formation *in vitro* entails an increase in low-angle light scattering. Buchthal, Knappeis and Sjöstrand actually found such change in muscle in the early stages of excitation preceding contraction. The relation to the increase in transparency, observed by D. K. Hill, is less clear.

Buchthal with Folkow (1944), Kahlson (1944–46), and Knappeis and Munch-Petersen (1946) elicited contraction with ATP on freshly isolated fibers in an isotonic milieu. This has no direct bearing on the present problem, the effect having been produced by an action on the membrane, and not by a direct action on the contractile matter. According to Beznák, ATP in this case acts by eliciting acetyl cholin formation.

The reader may object to our having dropped the G-F transformation of actin altogether. This change is so remarkable that it must have its place in the cycle. Certainly, it must; the question is: What is it? There can be little doubt that happenings in muscle depend on the forces developed by the actin particles. These in turn depend on the way in which the actin globules are fitted together to form ovoids, a process governed by ionic balances. Therefore any change in the ionic balance must act on the interrelation of globules and thereby influence the outside field of forces surrounding the globule. There is, however, no need for the process to go to such extremes as the complete disintegration into globules observed under extreme artificial conditions such as in the complete absence of salts. The F-G depolymerization is thus but the extreme case of a most important process.

3. Contraction

"Excitation," in its effect, means actomyosin formation. In the presence of ATP a deep-going inner depolarization of charges takes place inside this complex declaring itself by the loss of hydration. According to the theory presented, this complex is unstable in its extended initial state and tends to dissipate its thermodynamic potential, going over into its energy-poorer shortened form. The difference in length and energy together can be made to do work, for instance lift weight. Some of the energy will have to be used to overcome inner friction and may be responsible for Hill's heat of shortening (1949). If shortening could really be prevented and all parts of the muscle fiber would remain isometric, probably no energy would be spent at all and the

complex could dissociate again without splitting ATP, provided that meanwhile the resting ionic balance was restored. In reality such ideal isometry does not exist, especially in a twitch in which the contracting parts can stretch the uncontracted ones. Q-bands stretch I-bands and also stretch the tendon to some extent so the system has to complete the full cycle before returning to its initial state.

4. Relaxation

When trying to interpret relaxation we are in much the same position as the man who when asked to repair a motor has to confess that he does not understand how a motor runs at all, therefore he cannot say why it stopped. In order to explain relaxation, we first have to achieve a better understanding of contraction. Nevertheless we can try to continue the story which we left with actomyosin-ATP in the contracted state.

Since ADP does not elicit contraction, it seems likely that it cannot maintain the contracted state either. Thus dephosphorylation of ATP to ADP could bring about relaxation which could persist if the wave of excitation has passed and the resting ionic balance has been restored. The bond energy of the ~P takes care of the energy balance sheet.

We do not require the muscle to stretch out actively to its initial length. We are content if it offers no considerable resistance to being stretched by its antagonist. Whether this is achieved simply by the dephosphorylation of ATP to ADP or only by the subsequent dissociation of actomyosin into its two components, we do not know. We also do not know whether ADP is capable of inducing this dissociation or has to be rephosphorylated to ATP before it can do this. In any case we can expect the ADP to be rephosphorylated promptly as long as CP and creatine phosphopherase are present. Since we started with actomyosin-ATP, it is this rephosphorylation which completes the cycle. This sequence agrees with Dubuisson who places hydrolysis of CP into relaxation (1947).

If contraction of actomyosin is essentially identical with its superprecipitation, then relaxation is identical with the redissolution (and dissociation) of the superprecipitate. It is easy to show that the active collaboration of ATP is needed for this latter process. If actomyosin is superprecipitated and the precipitate is separated in the centrifuge and suspended in hypertonic salt solution, it will not dissolve in the absence of ATP, but will do so immediately if ATP is added, provided we do not allow too much time for hysteresis, which may make itself felt within seconds, to occur. Whether ADP would do the trick has not yet been ascertained.

A final remark concerns the bond energy between ATP and myosin.

ATP has a great affinity to myosin and is linked to it strongly. This means a high bond energy. Mommaerts has tried to calculate this energy and found it within the range of 10,000 cal and thus almost equal to the free energy of the P—O—P link. If we had to take this energy into account, the situation would become very complicated. If, however, we do not remove that ADP from myosin, the whole complication drops out. If ADP is dephosphorylated and the AMP has to be removed, the energy of deamination may pay for the bond energy.

We can now sum up the whole story by means of symbols used earlier, marking the presence of a high-energy link with an exclamation mark, the relaxed state with *R*, the contracted state with *C*, and the wave of excitation with a wavy line.

Rest: actin+myosin-ATP!

⇅

Excitation: actomyosin-ATP!$_R$

⇅

Contraction: actomyosin-ATP$_C$

⇅

Relaxation: actomyosin-ATP ⇌ actomyosin ADP+phoshate

actomyosin-ADP+CP ⇌ actomyosin-ATP!+C

⇅

actin+myosin-ATP!

Naturally, this is merely a theory, and the reader is warned that the author had made two theories before, at least about mechanics, both of which are definitely wrong. The chances that a theory is right increase in proportion to the difficulty of fabricating it and as more and more facts, which have to be taken into account, become known. The difficulty of the job has lately been increased by the EM and X-rays. All the same, a theory is only a theory, and alternate theories can be built.

5. Extracted Muscle

Extracted muscle behaves differently. The close and regular packing of this material strongly favors hysteresis which joins actin and myosin permanently. While intact muscle does not contract spontaneously because it has no actomyosin, extracted muscle does not contract because it has no ATP. It does so if this substance is added, its proteins being in the same state as they are in intact muscle in excitation, i.e., associated to actomyosin. As this association is permanent. there will be no relaxation with this material. As long as ATP is present, it will be contracted, and if the ATP is eliminated, it will

freeze up (Fig. 49). With this material we can thus study contraction uncomplicated by relaxation or excitation (Korey).

On the addition of ATP the fibers will contract not only in hypotonic salt solution but they will also contract even above isotony (0.16 *M* NaCl or KCl) at any salt concentration up to 0.5 *M* (above which the actomyosin dissolves in the presence of ATP). Dissociation of actomyosin can be enforced in this material by the use of drastic agents such as KCN, pyrophosphate, or $NaHCO_3$ (A. G. Szent-Györgyi) after which the muscle will once more refuse to contract at higher salt concentrations. If placed in hypertonic salt solution in which no contraction takes place in spite of the presence of ATP, freezing will bring the dissociated actin and myosin together, and on thawing the muscle will contract, having been "excited" by the cold (A. G. Szent-Györgyi).

Roy showed that hysteresis in washed muscle occurs within seconds. A fresh homogenate of muscle will have its maximum superprecipitation in the presence of 0.1 *M* KCl and will not superprecipitate above 0.16 *M*. If prior to homogenization the muscle is "washed" for a few seconds in water, maximum superprecipitation will be found at 0.3. Fresh muscle is glassy and transparent, washed muscle white and opaque. Transparency could be restored by enforcing dissociation of actomyosin (A. G. Szent-Györgyi). All this also explains why Biro and Szent-Györgyi (1948) produced a loose hydrated mass when washing muscle strictly at 0°C and a dry-looking, hard, dehydrated mass when washing 2 degrees higher.

Smooth Muscle and Heart Muscle

However different in size or form, all muscle cells seem to tally in one respect: the contractile matter is actomyosin and is more or less closely related to the actomyosin of cross-striated muscle in the rabbit. Rozsa (1946) has shown that the actin and myosin of body, heart, and smooth muscle of the rabbit are interchangeable, actin from one giving contractile actomyosin with the myosin of the other, and vice versa. The actin and myosin prepared from the stomach of the goose acted in the same way. Ciganda, Citterio, Ranzi, and Tosi showed that actin and myosin could be interchanged if prepared from rabbits, pigeons, frogs, and bony fish. Languste myosin could not be combined with vertebrate actin, but actin from the same animal worked with myosin of the rabbit, pigeon, or bony fish.[1]

Lajtha (1947) has studied the myosin of molluscs (clams). He prepared actomyosin, made it contract with ATP, and found its behavior to be similar to that of mammalians. Sarkar (unpublished) prepared glycerinated *Limulus* muscle, studied the temperature dependence of its ATP contractions, and found relations similar to other animals located much higher up the evolutionary scale.

Naturally, this does not mean that there is no difference between muscles of different animals, or between body, heart, and smooth muscle. The difference in function and structure are obvious and are most important both from a physiological and from a medical point of view. These differences, however, could not be studied before the basic properties of actin and myosin were established. For this pur-

[1] As shown by Guba (1943), there are big differences in the extractability of actin and myosin in different animals. In several species of fish, for instance, actin dissolves very easily and even a short extraction yields an actomyosin with a high actin content. There are considerable differences also between different muscles, such as heart and body muscles, in one and the same animal. In the rabbit conditions favor the preparation of actin-free myosin, since its myosin goes into solution fairly easily and actin rather slowly.

pose cross-striated muscle, with its rapid function and considerable mass, was the most suitable material. Now that the outlines are known, the way is open for studies in this direction.

Extending our studies from cross-striated muscle to other material, our first question is how far the peculiarities observed are due to differences in the contractile proteins themselves and how far to superstructures such as the apparatus of excitation and its propagation. If the difference lies in the contractile matter itself, then we might ask how far they are due to the contractile proteins themselves, to actin and myosin, and how far to regulating substances attached to these proteins. Snellman and Gelotte (1950b) find myosin prepared from heart muscle different in its physico-chemical behavior from myosin prepared from body muscle.

The first steps made toward these problems show their great fascination and medical and physiological importance. I am referring to the studies of Csapo on the behavior of the uterus muscle in pregnancy. In some ways the uterus muscle is a wonderful material for the study of the generation of contractile proteins, because the quantity of actomyosin increases sixty times during pregnancy and decreases in the same proportion after it.

As shown by Csapo, the uterus is relatively poor in actomyosin before pregnancy, especially in actin; its actomyosin thus shows little contractility. Not only does the quantity of these proteins increase during pregnancy, but also the actin/myosin ratio, leading to the production of a very reactive actomyosin at the end of the term, when the muscle has to enter into activity. The distribution of actomyosin between corpus and cervix also follows the physiological function. In the earlier stages of pregnancy, when cervix and corpus have to bear the strain equally, the actomyosin content increases in both, while at the end of pregnancy the cervix drops behind, the corpus prepares itself for its active role and the cervix for its passive role in delivery.

The threads prepared from actomyosin extracted from the uterus contracted very slowly under the influence of ATP as compared to the contraction of threads prepared from actomyosin of cross-striated muscle, which reflects the great difference in function, slow in the uterus and rapid in body muscle. The contractility increased during pregnancy, to reach maximum in labor. All these impressive changes are regulated by sex hormones and are a most wonderful material for the study of the chemical mechanism by which hormones exert their activity.

The sex hormones producing these changes are steroids, but it seems probable that the influence of steroids is not limited to this

case; it seems more likely that all muscles depend equally on steroids for their development and that the regulation of uterine changes is effected by making this organ dependent on a specific steroid produced by a specific secretion.

These steroids are not the only low-molecular substance involved in building and regulating muscle. We have discussed earlier the construction of actin ovoids, in which process ATP played a leading role. Probably it acted by temporarily depolarizing certain forces and providing others needed for fitting the globules together in the right way. There is no reason to believe that the role of ATP is finished once the ovoids have been constructed. It is probable that owing to heat agitation and molecular accidents a certain measure of demolition and construction is going on all the time within the living structures.

The role played by ATP in the construction of the actin ovoid is but one instance of directing forces being provided by small molecules in the construction of biomolecular structures. Different hormones may have an analogous function. Analogous processes of destruction and construction are probably going on in all tissues permanently. Such a role may perhaps explain why the action of certain hormones has a lag period, ranging from hours to days, before becoming manifested. It may explain the striking disproportionality in hormonal action and the lack of all stoichiometric relation and may also explain how very small quantities of hormones can have a decisive influence on relatively large masses of structure which disintegrate if the hormone is not available.

Not only the construction but also the actual working of the system may depend on the presence of small molecules. In our discussion of thermodynamics we have encountered crystalloids modifying the action of muscle by changing one or the other of the basic thermodynamic constants, ΔF, ΔH, or ΔS. Better knowledge of these regulatory substances may not only shed light on the mechanism of their action, but may also enable us to take the regulations into our own hands and correct nature's occasional shortcomings.

Empirism has also provided us with substances which, though not normal constituents of tissue, can exert a strong regulatory influence, helping us even when Nature itself fails. I am alluding to the group of digitalis glycosides which have a specific influence on the heart. The question presents itself how these substances are related to our experience with muscle. Horváth, Király, and Szerb have found that digitalis glycosides (strophantin G and digitoxin), in quantities of gammas, greatly promoted the G-F transformation in actin, but

only if this actin was prepared from heart muscle. The same drug was devoid at the same time of any activity if applied to actin of body muscle even in a hundred times bigger concentration. As we have seen, the G-F transformation is the extreme of a most important interrelation which decides the forces developed by the actin ovoid. Snellman and Gelotte (1950a) found that actin prepared from heart muscle was accompanied by an ATP desamidase which prevented actin polymerization by destroying the ATP present. This enzyme could be inhibited by very low concentrations of digitalis preparations.

The first steps in immunology were made by Keztyüs, Nikodemusz, and Szilárd who showed that while myosin is heteroantigen, actin is isoantigen, that is, the injection into the animals of their own actin and myosin produces antibodies in the first but not in the latter case. Actin is organ specific, myosin is specific for species. Myosin will produce a general reaction only if introduced into the bodies of animals belonging to a different species. Actin will do so whenever it gets into the circulation of the very same animal. This finding can become of primary interest for medicine.

It is easy to believe that the little actin globule can pass the blood stream barrier and get into the circulation if, for some reason, the architecture of actin (or an analogous protein) gets out of order. Arrived in the blood it will produce general symptoms. Diseases like rheumatic fever may be due to some such cause, and the author regrets that the opportunity is denied him to extend his experiments into this field.

Of all the single muscles, the heart has claimed the most interest for it is but a bag made of muscle. Medicine has so far not been very successful in the understanding or the correction of its pathological changes, though it has greatly refined the instruments of clinical diagnosis. The situation has not essentially changed since my student days when somewhat sarcastically we said, after examining our patient: "Therapy: X-ray and EKG." This state of affairs is natural. We cannot understand the heart till we understand what muscle is, with all its subtle regulations. Until this is achieved, the heart and those little muscle cells in the precapillary arteries must remain the main killers of man. The realization of our ignorance in this field came as a shock to me and is one of the deepest impressions of my scientific career. With the discovery of the contractile complex and its reaction with ATP, the way is now open for a more profound analysis.

In the analysis of a pathological condition, let us say a weakness of muscle, be it heart or body muscle, the first step is the localization of the trouble. This may lie outside the muscle, in the nervous mecha-

nism, or it may lie on the path between the nerve and the contractile matter, in the membrane, or in the mechanism propagating the impulse from the membrane to the contractile matter. If the trouble is traced to the interior of the muscle fiber, then the question is whether it is located in the contractile matter itself or in the energy-producing apparatus which is responsible for oxidation and fermentation and which provides the contractile matter with energy, that is, with ~P. If the trouble is finally traced to the contractile mechanism itself, then the question arises whether the trouble is with the proteins themselves or with some other substance involved in the regulation of the function of these proteins. We may approach the problem also from a functional angle. In the author's opinion the thermodynamic analysis opens a road quite as hopeful as morphology. Glycerol extraction of muscle offers a method for conserving the material and studying at ease the dynamic properties of the contractile matter.

Before closing this chapter, mention should also be made of studies of Kováts who found the actomyosin content of different parts of the heart is proportional to their mechanical activity. The author also wants to mention a few of his abortive attempts to test ATP, the master substance of muscle, for its therapeutic applicability. Since he had found that under certain conditions an increase in ATP concentration favors relaxation, while a decrease favors contraction, the author advocated the trial of ATP in pathological conditions characterized by a spasm of muscular elements. Such conditions are angina pectoris with its spasm of the small coronary arteries, vasospastic gangrenes of the Burgi type and dysmenorrheas. Body muscle contains ATP in a high concentration and so a slight increase in its quantity cannot be expected to have any direct effect. The heart muscle contains less, therefore it is not surprising that Drury and the author should have found ATP and related substances pharmacologically active in the heart. The ATP concentration is the lowest in smooth muscle and thus the strongest effects could be expected in this tissue. The results obtained at the Medical and Obstetrical Department of the University of Szeged, where these experiments were begun (and discontinued because of World War II) were favorable. Pettko and Straub's and Lichteneckert and Straub's experiments should be mentioned, according to which the heart of summer frogs, arrested by chloroform or quinine, can be restored to normal activity by very small quantities of ATP, in spite of the presence of the poison. The author also recalls his experiments with Drury in which ATP was found to have a striking effect on the rhythm of the heart, and which seemed to advocate a serious trial of this substance in arhythmic conditions.

There are reasons to hope that the experience collected on body muscle will not only help to understand other sorts of muscle, but may help to understand apparently entirely different organs and functions.

Fresh muscle, if extracted with strong salt solutions, yields a viscous solution. This high viscosity is due to the presence of the nucleotide ATP with its ~P and the consecutive dissolution of the structural protein (myosin). If the minced muscle is stored for a few hours prior to extraction, an extract of low viscosity is obtained. The difference is due primarily to the decomposition of the ~P, in the absence of which the structural protein does not hydrate and solvatize. As shown by Banga and the author (1940–49), all organs, if freshly minced, yield highly viscous extracts with a strong salt solution. Lajtha (unpublished) studied this phenomenon in more detail in kidney and found that, as in muscle, after a few hours of storage, kidney also yields an extract of low viscosity. Parallel to this change labile phosphate disappeared. This labile phosphate was water insoluble, linked to structure, and thus probably to nucleic acid, supporting the author's contention that ATP in muscle fulfils an analogous function to nucleic acid in other protoplasmic structures. Lajtha also found a labile phosphate in the isolated nucleic acid of kidney, one for every tetranucleotide, but was unable to establish any direct relation between this phosphate and the change in viscosity.

Runnström and Kriszat have shown that ATP induces changes in the physical state of egg cells and Kriszat has shown the same to be true of amebae.

All this suggests that the blueprint of the activity of the different sorts of muscle is *mutatis mutandis* also the general blueprint of biological action.

The author is aware that all these remarks on pathology and comparative chemistry are very fragmentary. This incompleteness only demonstrates the freshness of this almost virgin field now laid open, promising a rich reward to the researchers who venture into it.

The Continuum Theory

When the author was writing his earlier book on muscle, he hoped that in his next one the chapter on the continuum theory would be the most important and extensive. Much to his disappointment this is not so, and the chapter even shows considerable "volume constriction." Nevertheless, the problem is one of the basic and most important ones and is still there, unsolved. It is this: when we put a few dozens of atoms together into an amino acid, an entirely new system is formed, with entirely new qualities which in no way resemble the qualities of the four parent substances: carbon, hydrogen, nitrogen, and oxygen. The amino acid has qualities of its own which can be expressed only in terms of amino acids, and we would be wrong if we visualized the amino acid molecule as an agglomerate of carbon, hydrogen, nitrogen, and oxygen.

Out of the two to three dozens of different amino acids Nature builds a protein molecule, the main bearer of life and instrument of its subtle reactions. We can take it for granted that once again something entirely new has been constructed, entirely new qualities have been developed. The problem is: what are these qualities, what is this wonderful new unit? Most biochemists dislike unfounded phantasies and like to build on solid and familiar ground, therefore as far as attempts have been made to interpret the qualities of proteins, they were mostly made in terms of atoms, atomic groups, charged points, and amino acids. Yet in trying to avoid speculation, unnoticed the most phantastic and unfounded theory was produced—that a protein is nothing but a more or less orderly heap of atoms or atomic groups, or amino acids. Though never formulated, this theory thwarted the road to the understanding of life during the first half of this century and is responsible to a great extent for the fact that not a single biological function is understood. To avoid making theories may be equivalent to making them.

Indications are numerous that a protein particle is a unit *sui generis*

and that there may be actions far exceeding interatomic distances, and these have been obtained in very different ways. One of these is connected with the idea of a "treffer" area, the problem being to determine the size of the field in which one single treffer by a photon may cause damage. Timoffeef-Ressovsky, Zimmer, and Delbrück used the damage done in the genetic apparatus by an x-ray treffer to obtain information about the size of the area in which such an event could cause trouble and found it considerable, far exceeding atomic distances.

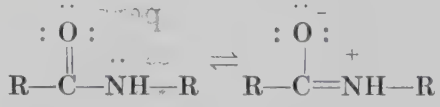
Büchner and Kaspers have shown that the light quanta absorbed by the protein moiety in myoglobin knock CO from the haem-partner of the complex with a quantum efficiency of unity, showing that the energy adsorbed by the protein can be propagated through the whole system on to the prosthetic group. Study of photosynthesis has shown that somehow the single quanta must be collected from a larger area and channeled to one spot to reduce one molecule of CO_2.

Scheibe has demonstrated the possibility of propagating energy across a system of cyclic molecules arranged parallel. Living structures are always nucleoproteins, and nucleoproteins contain cyclic formations which, as shown by Butenandt, *et al.*, are arranged in a very regular fashion. Astbury (1947) has shown that the periodicity of the single nucleotides is the same as that of the amino acid residues in a protein. Myosin has no nucleic acid, but in intact muscle there is one ATP molecule for every 18,000 g, and if these were arranged properly, they could provide for continuity. When studying the relation between contractility and ATP concentration in muscle, Borbiro found that excitability in muscle decreased with the ATP concentration and ceased entirely when the ATP concentration dropped to half its normal value, that is, every second ATP dropped out. Talmud's theory may also be recalled in this connection, according to which the cohesion between the homoiopolar cyclic side chains of amino acids are responsible for the stability of the protein molecule. This would mean that these cyclic formations are linked together, perhaps allowing transmission of energy across the planes of these cycles.

These observations do not tell us what the underlying electronic structure is or in what form the energy is propagated; they merely show that actions reach beyond atomic distances and that there may be some sort of continuity within the larger system. Such an assumption is also necessitated by various biological phenomena. It is impossible, for instance, to explain the interaction of cytochromes, the migration of electrons from one to the other without supposing some sort of a mobility in these electrons, for these cytochromes are linked to structure and are thus fixed in space. It is very difficult to imagine steric rela-

tions which allow an electron to jump from the Fe of one directly to that of the other. In fact, these cytochromes show analogies to metallic impurities of crystals in which electrons can drop to lower energy levels. Some such thing may occur in cytochromes, this drop in energy being coupled with the establishment of high-energy phosphate links.

In order to discuss the possible underlying electronic structures adequately, the author would have to exceed both the limits of this book and the limits of his own knowledge. There are many possibilities. The author himself speculated some time ago (1941) along lines of energy band spectra, supposing that the regularity in a protein molecule was sufficient to make the outer electrons of the peptide chain crowd in common energy levels, in more or less continuous bands. Suggestions along these lines have also been made by P. Jordan. The author's associate, J. Gergely, later joined M. G. Evans of Leeds to study under his guidance properties of electrons within the system of H-bonds, supposedly established between the CO and NH_2 groups in protein. Working with the molecular orbital method, they found that such a system of H-links corresponded to an energy minimum and thus had a certain stability. They found that certain electrons within this system have a tendency towards non-localization and have a corresponding mobility, comparable to that of the π electrons in benzene. The electrons were found to be crowded into saturated bands, the highest being separated from the next empty one by a distance of several volts, which agrees with proteins being colored in the ultraviolet only. Wirtz discussed similar possibilities earlier dealing with the H-bonds as with building stones of one single system in which every new member contributes to the stability of all the rest. W. Schmitt extended these ideas, invoking transposition of bonds as intruments of energy transmission. Denbigh brought forward the idea of two resonating forms of protein corresponding to:



Somewhat similar ideas were expressed earlier by Huggins. Proteins have also been shown to have a high polarizability along the peptide chain, and it has been shown that the C—C distance is reduced in the middle C—C bond between two 1–5 double links (Bateman and Jeffry). Proteins contain C=O links at this distance. Hyperconjugation has also been quoted and O. Schmidt has attempted to explain nervous activity with such ideas.

A few abortive experiments were also performed in the author's laboratory (1) to obtain experimental evidence on these questions. Phosphorescence of so-called gelatine phosphores was studied in this connection by Gergely (I) but, as pointed out by Kasha, this has lost its meaning, since it has been shown meanwhile by G. N. Lewis that phosphorescence in such systems is not connected with a band structure. Since they were made during World War II, these studies of Lewis were unknown to the author. An attempt was made by *Boros* to demonstrate an internal photoelectric effect in similar systems. Results were encouraging, but in the end the photoelectric conduction observed could not be separated with certainty from thermal effects, and the experiments remained inconclusive. A study of the photoelectric effect in proteins induced by ultraviolet seems to be more promising, but is at present outside the author's experimental possibilities.

There is little doubt in the author's mind that progress along these lines, the application of quantum mechanics, and the theory of the solid state will be the next milestone in the history of biochemistry. Biochemistry at present is built on the idea of the covalent bond, it is a science based on primary valences. The subtle changes within the living structure are connected with the breaking and forming of primary valences only in a remote, secondary way and are by no means explained by the fact that the protein molecule, when boiled for twenty-four hours in concentrated hydrochloric acid, disintegrates into amino acids. The peptide chain may be the backbone of the structure, but it tells us no more about the real function of the protein then the dug-up backbone of some prehistoric animal tells us about its married life.

Whether muscular contraction can be understood without some sort of an energy continuum within the protein, solely by a localized action at single points, is an open question.

An attempt to explain contraction in muscle as a localized action was made by Riseman and Kirkwood, who based their calculations on changes in the elastic modulus, and asked if ATP induces a local change in charge of one elementary unit, by its dephosphorylation, what would be the relative distance and the number of such changes which could answer for contraction. They arrived at the conclusion that the single point of action should be about 100 A apart and there should be about 100 of them in every myosin molecule of MW 10^6/g. One hundred angstroms is not an unreasonable number, but 100 ATP's per mole of myosin is beyond practical possibilities. If would mean 14 charges and thus 14 moles of ATP per 70,000 g of myosin, whereas muscle contains no more than 4, all told. The 14 charges demanded by Riseman and Kirkwood correspond to the over-all charges of myosin. Myosin in salt-free solution has 14 over-all charges per 70,000 g, mainly due to the dissociation to the imidazole group. They can be depolarized by ions, but could also be depolarized by the opposite charges of actin

when actomyosin was formed, thus opening the possibility of explaining contraction by means of local changes in charge due to the mutual discharging and precipitating action of the two proteins.

As has been mentioned before Mihályi's preliminary measurements indicated that actin has three times as many charged points as myosin for the same weight, and it has also been mentioned that one part of actin unites with three parts of myosin to form actomyosin. This suggests that superprecipitation may be a mutual discharge of all these groups, provided their pattern fits. The role played by ATP would thus remain obscure, but contraction in this case would be explained by the interaction of single points.

Observations are also available indicating that the protein molecules in biological reactions react as a whole. The simple fact that protein denaturation is mostly an all-or-none reaction is a strong suggestion in support of this. That muscular contraction is brought about by the action of the whole protein particle was suggested by the "Ebbecke phenomenon." Ebbecke showed that under high pressure muscle contracted, the contraction being a function of pressure. D. E. S. Brown (personal communication) showed that contraction is connected with a decrease in volume of the myosin particle and that contraction and volume decrease are an equilibrium reaction which can be treated thermodynamically and which even allows us to calculate the external work done by the system. Observations have also been made by Brown suggesting that the volume changes taking place in actin during contraction are the opposite of those of myosin. All this strongly suggests that the particles of these proteins act as a whole. Work and efficiency can be calculated from the volume changes, and thus work, contraction and the volume change must be merely different expressions of one and the same phenomenon. Possibly the volume constriction of muscle discovered by Ernst has the same foundation, a decrease in the volume of the myosin particle.

If the particle gives up its free energy as a whole, then the energy of ATP must be communicated to it as to a whole and must somehow be diffused from the point of interaction between ATP and the protein to the more distant parts of the latter. The importance of this problem far exceeds the limits of that of muscular contraction.

The author's long association with P. Gombás first in Budapest and later in Woods Hole, Massachusetts, has also led to pointing out a possibility (Gombás and Hoffmann) of explaining muscle contraction by a change taking place throughout the whole particle, corresponding to the two resonating structures of protein discussed by Denbigh (see page 132). In one of these two forms the N's and O's are charged,

while in the other they have no charge. Gombás and Hoffmann show that the charge of the N's leads to a greater mobility of electrons in the system with its H-bonds, while the discharge leads to a change in the valence angles entailing a shortening of the peptide chain by about 10 per cent and its curling or spiraling up, which may reduce the length by two-thirds. Naturally in this mechanism of shortening, actin and ATP also would have to have their place. Myosin without actin and ATP has but one state in which it is strongly charged and hydrated. Its discharge would have to be made possible by its association with actin and elicited by the ATP. Since the ATP can only act at one point, we would have to suppose that it acts at some special "strategic point" from which the action spreads over the whole particle. Different mechanisms could be used to propagate the change. The mobility of electrons could be instrumental or a "zipper-mechanism." While the attachment of ATP would tip the whole structure over into the discharged form, its dephosphorylation to ADP would have to tip it back.

This theory of contraction, making a charged and discharged state of the protein responsible for the dimensional change, is in good accord with earlier observations as far as it assigns to the elongated relaxed form a higher charge density and to the contracted state a discharge. We have seen that actomyosin in its relaxed state is very hydrophylous and loses all affinity to water in contraction; affinity to water is an expression of a charged, its absence an expression of a discharged condition. The theory disagrees with our thermodynamics inasmuch as it makes internal potential energy responsible for the work done, while thermodynamics and the Wiegand-Snyder equation, as applied by the Webers and by Gergely and Laki indicated a major change in entropy. Possibly the truth lies in the middle and the charged state is responsible for the hydration and thus for the linear configuration of the myosin particles, while the discharge makes this configuration improbable and leads to the statistical folding.

Whatever the mechanism of contraction, whether it is due to local actions or to different resonating states of the whole protein particle, the behavior of the contractile matter in muscle clearly reflects a basic regularity in living matter: that it has two states, the state of rest and the state of activity, the two being characterized by a difference in free-energy content. The active state is the state poorer in free energy, the $-\Delta F$ being used to perform the work demanded by biological activity. The nature of the work done depends on the specific structure, i.e., the specific function of the system. If this function is to produce motion, we can expect the peptide chains to be arranged in long, linear threads,

as is the case in myosin, which structure makes shortening possible. If the specific function were the pumping of water (as in the case in kidney) such a linear arrangement would not make sense and we could expect the differences in affinity to water to be operative and produce osmotic work. Since action in the primitive monomolecular organisms must be elicited by some impression reaching the cell from outside, from its surface, we can expect the whole machine to be set in motion, the whole exergonic reaction to be tipped off by some change, and a depolarization in the surface membrane, transmitted to the reacting machinery by the medium, an arrangement which need not be altered by the single cells joining to higher multicellular organisms.

In the descent along the scale of evolution and organization of primitive single organisms, the next step below cellular level is that represented by bacteria which have no membrane and consequently do not have two states. Bacteria mostly do only one thing, they grow and are permanently in the active state tuned for proliferation, which sets in as soon as foodstuffs become available. It seems logical that the next higher step in evolution from this state should have been the development of the membrane, which made it possible to perform more complex reactions by reserving the activity for and directing it toward useful purposes.

As we descend along the scale of evolution from the bacteria level, we could imagine bacteria first gradually becoming smaller, approaching the size of viruses and macromolecules. The typical viruses, however, lead us into a blind alley in our pursuit of the origin of life since they are dependent for their growth on the internal milieu of higher cellular organisms. A few remarks on their nature will be made in the next and final chapter of this book.

Water and Viruses

According to Sir Oliver Lodge, the last thing a deep-sea fish would discover is water. We are all deep-sea fish, with the difference that we have the water inside, not outside us. As pointed out on an earlier occasion (1939) all life, from an energy point of view, is nothing but a play of water, and we ourselves are but a pailful of seawater which has grown a skin to be able to crawl on land.

The final source of all energy that drives life is the radiating energy of the sun, which is picked up by the *chlorophyl*, the green dyestuff of plants, and is locked up by their photosynthesis into little parcels which we call " foodstuff-molecules." Photosynthesis, in its essence, is the separation of water, H_2O, into its elements, hydrogen and oxygen. A great deal of energy must be invested to effect this separation, and this can be recovered by reversing the process and uniting hydrogen and oxygen to form water again. What a plant does is to pick up the radiation of the sun, invest its energy in the separation of the elements of water, send the oxygen into the atmosphere, and store the hydrogen. Since hydrogen is a gas, it would be impossible to store it as such; therefore the plant picks up carbon dioxide, CO_2, from the atmosphere and sticks the H-atoms to it, as we hang a hat on a rack. The compound thus formed, consisting of C, H, and O, is our main foodstuff, carbohydrate. In order to release the energy of the sunshine enclosed in it and have our living machine driven by it, we reverse the process of photosynthesis, take the H-atoms off the C, pick up O_2 from the atmosphere, and join the two to form H_2O. That it is actually the electron of the H which yields the energy by dropping to the level of oxygen is a matter of detail. What interests us here is that the energy cycle around which all life runs is a play of water, a separation and reunion of its elements. The quantity of the energy bound and released in this process is rather great, some 68,000 cal, comparable to a large banknote. Our cells, in the daily life of their household, naturally need smaller change, therefore the first step is to exchange these banknotes for smaller coins of 11,000 cal. These

11,000 cal are the currency of the cell. Again it is the water molecule which is the bearer and instrument, but this time the investment consists of taking out a water molecule from between two phosphate molecules which thus become joined by a "high-energy phosphate bond," "~P" as Lipmann writes it. By returning the water and allowing the two phosphates to part, the 11,000 cal invested can be released again and used to cover the petty expenses of the household of our cells.[1]

In spite of the paramount importance of water, in spite of the fact that this water makes up four-fifths of our tissues—all of us being but a 20% watery solution—biochemistry took hardly any notice of it. Water was simply looked upon as an inert inner medium, as inactive as the nitrogen in our outer atmosphere. It is even said that one can drink water by the pint without it causing any harm.

In spite of this apparent inactivity, in the author's opinion water, in a way, is one of the most active substances on earth. I can imagine forms of life developing in sulfuric acid which would regard vitriol as the most inactive substance. On dissociation water yields H and OH ions, the most active ions we know. The specific heat of water is quite extraordinary. It is probably due to the properties of water that the temperature of all homoiotherm animals is adjusted to 37–41°C, which, as Barnes emphasized, is the temperature at which water has its minimum specific heat, indicating that this temperature is an important turning point for the properties of water.

The most striking quality of water is its high dielectric constant, which means that water greatly depolarizes the action of electric charges by being a dipole, that is, by having (or developing) a positive and a negative end and turning the opposite end toward any charge and building a structure around it. If the charge is weak, the "structure" may be no more than a certain statistical probability of the water molecules, to be found in a certain position. If the electric field is strong enough, the water will not care for another weaker charge and will not act as solvent for a third substance, but will be "bound water." As far as the electric forces reach, water will tend to depolarize them by building structures around them. These charges may be net

[1] Photosynthesis must have existed before Nature discovered chlorophyl and learned how to herd four light quanta into one single reaction, four quanta being needed for the reduction of one CO_2 (Warburg). Nobody knows what this "pre-chlorophyl photosynthesis" was. The author's guess is that carotenes or related pigments were involved, investing the single quanta directly into ~P's. Perhaps it is this course of events which is reflected by the predominating brown color of forests in the early spring before chlorophyl appears, or by the pigments of vegetable and animal skins.

charges, acquired by dissociation or by the adsorption of ions, or may simply be due to a defective inner balance, to the electropolarity of links. Whatever its origin, an electric force attracts the water dipole, and this attraction declares itself to the chemist as "hydrophility."

The nature of the water structure will also depend on its distance from the particle which generates it. In its immediate vicinity every charged point will have its own individual water sheet. As the distance from the particle increases, the effect of positive and negative charges compensate one another, and only the over-all charge makes itself felt; its action may reach as far as several hundred angstroms. It is known that tobacco mosaic virus particles arrange themselves in water in a regular hexagonal array even if they are as far as 300 A apart (Bernal and Fankuchen). This can hardly be explained otherwise than by the water structures surrounding the single particles making one single unit with them, which units tend to assume the closest packing. Similar directive influences have been found in inorganic tactoids reaching as far as 1000 A.

The ions which hold the over-all charge in balance must be located in this water sheet. These ions will also have their own water sheet, and the whole picture will be further complicated by the strong tendency of the water molecules to associate among themselves.

The first monolayer of water around the colloid particle can be expected to be the most strongly bound. Boyes-Watson, Davidson, and Perutz in their X-ray studies found the hemoglobin molecule coated with one monolayer of "bound water" the weight of which was about one-third the weight of the particle.[2] The screening effect of this layer will make the binding of the next layer much weaker, but Harkins found that the energy needed to remove a water molecule was still appreciably increased even in the fourth layer, compared to free water. The relative quantity of water bound by the very thin and long myosin particle with its high charge density must be considerable.

If two colloidal particles approach each other it will be the water structures which meet first and repel each other if they are of equal sign, or destroy each other if they are of opposite sign. Colloidal chemistry, repulsion and precipitation of particles, can all also be expressed in terms of these water structures, of their building up, and their collapse. These water structures have their own entropy and

[2] This strongly bound water in the immediate vicinity of the particle will not act as solvent for other molecules and consequently will protect the protein from their action. Such water sheets may play an important role in deciding permeability and may perhaps explain why K, with its smaller energy of hydration, is admitted by cells in preference to Na with its relatively much higher energy of hydration.

energy values, and it is difficult to say how far the changes in energy and entropy observed in such colloidal reactions as muscular contraction are due to the reactions of the particles themselves or to the reactions of the surrounding water structures. Since they form one single unit, the two cannot be separated, the water structure and the particle which generates them. This is also true in relation to life, and the discussion whether from the point of view of life the particle itself or the surrounding structure is the more important would have the same merit as a discussion on whether the zebra is a black horse with white stripes or a white horse with black stripes.

The nature of the forces surrounding colloidal particles and determining their interaction are still the object of discussion.[3] The discussion is more on the relative importance than the nature of different forces. There is a fair amount of agreement on the basic question that the relation of two colloidal particles is determined by the balance of repulsive and attractive forces, attractive forces being mainly of the van der Waals type, while repulsions are coulombic, that is electric in nature.

One may object to the idea of a force like the van der Waals force (which decreases very rapidly with distance, its sixth power) being able to balance a force which decreases only with the second power of the distance. The two get even by the first not being depolarized by the water and its ions, while the second is thus also decreasing rapidly with the distance. The situation is undoubtedly complicated even in the simplified case of two charged points or plates approaching each other from infinite distance. The situation becomes utterly complicated if a great number of particles is crowded together in a regular array in a limited space at very close range, as is the case in our actomyosin tactoid. Here the water and ion structures of neighboring particles must merge to form the only continuous phase in the system, comparable in its structure to a honeycomb. This structure itself may be the seat of most important biological activities such as, for instance, transmission of impulse. This structure actually decides the equilibrium distance between the actin and myosin particles.

Whatever the role of these water structures in contraction, they must have a decisive influence on the stability of the protein. Charges are the main instrument of life, holding the living structure together, but at the same time they are also its enemy, tending to pull the specific structure down, bringing opposite charges together, and bringing the protein herewith from its improbable native state into the more probable denatured state. Needless to stress thus the importance of

[3] Readers more profoundly interested in these are referred to Verwey's and Overbeek's monograph.

the protective effect of the screening water sheet and the consequences of its disturbance by drying or anhydrous solvents like alcohol.

As pointed out before, the nature of the interrelation between particles and the water also depends on the distance and will thus be different inside and outside the actin ovoids. Inside the ovoid, the distance between the single globules can be only very small, whereas outside, between the single ovoids or between ovoids and myosin, distances may be greater. What is of special importance here is that however different its structures, water will form one continuous phase from the membrane of the fiber right into the interior of the actin particle, making one single, unique system out of the whole structure, membrane, actomyosin, and all, one unique system characteristic of this cellular level of organization.

Modern physics tends to distinguish between states of matter with a regular or random structure rather than between solid and liquid. Regular structure is linked to the solid state. From this point of view the water of these living structures ranges with solids rather than with liquids.

The elucidation of all these regulations, the role played by water in life, in short, the discovery of water as part and parcel of the living machine will, in the author's view, be one of the major contributions to biology in the decades to come.

I cannot put down my pen without drawing attention to a rather remarkable analogy between the actin ovoid and certain viruses. Viruses are characterized by a certain size, shape, lack of metabolism, and ability to multiply only inside a cell. All these properties are shared by the actin ovoid. The actin ovoid shares with the poliomyelitis virus even its size and shape, its ability to form filaments, and its trypsin- and aceton-resistance. This may suggest that the polio virus is nothing but a degenerated form of actin or another analogous protein. It is a matter for rather fascinating speculation to conjecture what would happen if, for some reason or other, the actin particle should loosen its structural relations, emancipate itself from the higher cellular architecture, and, owing to the small size of its molecules, should pass through the capillary wall and enter a foreign cell, e.g. a nerve cell, and continue its proliferation. Probably it would act as a virus.

In the author's opinion this possibility of virus-genesis has not yet received sufficient consideration and if he has mentioned it here, this was not merely in order to add one more to his theories or speculations, but because he thinks that research into this question may lead to important practical results and help to relieve or prevent a great deal of human suffering.

Appendix

Methods

No exhaustive treatise on technique will be given here. Only a few remarks will be made, based mostly on personal experience, in order to facilitate work along these lines.

ATP

Lohman (1934–35) and most later workers extracted the ATP from tissues by means of tricholoracetic acid and purified the product by Ba and Hg precipitation. Dounce, Rothstein, Thannhauser-Beyer, Meier, and Freer claim that by using these precipitations in special sequence very pure preparations can be obtained. ATP, even in dry condition, is not quite stable and appreciable decomposition of its Na salt occurs at room temperature within months (Bailey, 1949).

A cheap and easy method for the preparation of crude ATP, also allowing production on a big scale, was described by the author (1947).

The most generally used method for ATP estimation is based on 7 minutes acid hydrolysis in 1 *N* HCl at 100°C, which splits off both labile phosphates. For the estimation of P the Fiske-SubbaRow method is mostly used with various modifications. Berenblum and Chain described a more direct method which allows the accurate estimation of very small amounts of phosphate. Borbiro and the author, starting from Berenblum and Chain's observations, described a P estimation which is less sensitive but allows the estimation of labile phosphate also in the presence of relatively large amounts of free phosphate or in the presence of pigments.

ATP, added to a 0.5–0.6 *M* KCl solution of actomyosin, causes dissociation of the complex and therewith a fall in viscosity. When the ATP is split by myosin, the viscosity rises again. This reaction has been used by Csapo to estimate ATP by comparing the action of an unknown solution with a known solution of ATP. The same principle has also been used by Kramer, Pettko, and Straub (see also Straub and Feuer, (1950). This reaction does not permit distinguishing be-

tween ATP and ADP. If myoskinase is present, the actomyosin will remain dissociated until both labile phosphates have been split off. In the absence of kinase it may remain dissociated indefinitely. The author used an analogous method earlier, producing superprecipitation instead of dissociation. If the actomyosin is free of kinase being prepared of crab meat (Lohman, 1935) or carefully washed myosin, ATP only causes superprecipitation, and the reaction may be developed into a differential method of ATP and ADP estimation.

Specific ATP estimations can be built on specific enzymic reactions, such as the transfer of the terminal phosphate to hexose by hexokinase. Hexose phosphate can then be estimated spectroscopically through its interaction with coenzymes (Kalckar, 1947).

The direct spectroscopic estimation of ATP is also extensively used (Kalckar, 1947) mostly in the Beckman ultraviolet spectrophotometer. It allows the differentiation between ATP and ITP and the detection of ATP even in the presence of protein.

Commercial ATP preparations often contain heavy metals, bound as a complex and inaccessible to SH_2. Their presence is revealed by the inhibition of the ATP-ase action observed if ATP is used in higher concentrations. The metals can be partly removed by adsorbing resin through ionic exchange.

The Glycerol-Extracted Psoas

The rabbit is killed and eviscerated. The psoas is exposed by cutting away the sidewalls of the abdomen and is liberated by placing the index finger under it and moving the finger in a cranial and caudal direction. Then the muscle is lifted slightly and punched through with small forceps with closed and pointed tips in such a way that the instrument separates a small bundle about 2 mm in diameter from the main bulk of the muscle. The bundle is now separated along its whole length by moving the forceps parallel to the fibers. A small stick (application rod) is placed alongside, and the fiber bundle is tied to it at both ends. Since the muscle is poor in connective material, it is easily cut through by ligaments. For this reason a relatively thick and soft thread (pearl cotton No. 5) is used in dry condition. Then the muscle is cut out, thus fixed to the stick at rest length. A medium-sized rabbit will easily yield fiber bundles 8 cm in length consisting of parallel fibers running continuously from end to end. If muscle of equilibrium length is needed, only one end of the bundle is tied to the stick, then the muscle is excised, straightened out, and its other end is also tied to the rod. On excision the muscle shortens by about 20 per cent, but only soon after death while the muscle is still warm.

The fiber bundles fixed to the rod are placed in 50% glycerol of 0°C. The easiest way to maintain this temperature is to place the glycerol in a fairly wide test tube and immerse the test tube in melting ice kept in a wide-mouthed thermos bottle. The thermos, if closed and placed in the refrigerator, will keep the temperature for weeks. The next day the fluid is exchanged for fresh precooled 50% glycerol. On the third day it is transferred to the deep-freezer at 20°C, where it can be kept for months with undiminished contractility. The glycerol prevents freezing and the low temperature stabilizes.

The author has less experience in keeping the muscle at 0°C, but he is told that even at this temperature the muscle will not lose contractility for weeks and remains in good shape. If the muscle strip is too thick, then its water is extracted before the glycerol can penetrate and the muscle becomes glassy and very hard. If detached from the stick it remains straight. It is nevertheless advisable to detach it only before use and have the muscle stored for three or four days in glycerol before the experiment. The microscopic structure of the preparation remains beautifully preserved.

The extraction of the soluble proteins from such fibers is very incomplete. As shown by Varga (1950) only about 50 per cent of the soluble proteins are extracted by glycerol. Consequently, the fibers contain all the auxiliary enzymes such as phosphorpherases and myokinase. Extraction with pure distilled water may be more effective, but if placed in water, muscle swells strongly, the swelling soon being followed by shrinking to the original size. In pure water contractility strongly decreases in a few days, even at 0°C.

The glycerinated fiber bundles are too thick to be used directly for experiments if the ATP contraction has to be studied. The ATP would produce contraction only on the periphery and might not reach the interior. This peripheral contraction would be inhibited by the uncontracted fibers in the middle. Using a 0.2% ATP, Varga (1950) found that fiber bundles containing five to seven fibers per diameter develop maximal tension. If this has to be measured at higher temperatures, it is advisable to soak the fibers in ATP at 0°C first, where the tension is relatively small, and then to transfer the preparation into the warmer ATP. Portzehl and Weber obtained higher tension in single isolated fibers. The tension observed in the author's laboratory is mostly around 1.5–2 kg per square centimeter.

In 50% glycerol the muscle is rather stiff and cannot be decomposed into smaller bundles or single fibers without straining them. Such strain later makes the fibers curl up if ATP is added. If the glycerol is completely washed out, the fibers become too soft. Best results were

obtained by placing the fiber bundles in 15% 0°C glycerol for an hour or so before decomposing them into smaller strips.

In decomposing the psoas into smaller bundles or isolated fibers, forceps with thin and well-fitting tips, like those used by watchmakers to handle fine springs, are very handy. With these one catches the end of two fiber bundles and simply pulls them apart. It is not difficult to secure isolated muscle fibers several centimeters in length.

The ATP contraction of muscle fibers is one of the most striking physiological phenomena and should be introduced into every course of biochemistry or physiology. It is also very instructive for the student, showing the mechanistic nature of contraction and is one of the few experiments which never fails. One single rabbit would provide a large class with material.

For ATP (0.2%) a commercial preparation may be used, but in class it may have more educational value to prepare a fresh-boiled juice of muscle. While the psoas is taken out and placed in glycerol, the rest of the body muscle can be minced and suspended in an equal volume of boiling water and quickly heated to 80–100°C. The muscle is strained through a cloth and pressed out, and its juice is used for experiment.

If the isometric tension of a glycerinated fiber bundle has to be measured, it is advisable to use a fiber bundle 4–8 cm in length. The instability caused by the great length of these very thin bundles may be reduced by clamping both ends in the same fine artery clamp between filter paper. The loop thus formed is placed around a glass hook. In this way the thickness of the muscle is doubled and its length halved without increasing the diameter.

Myosin

The preparation of myosin consists of the following steps: extraction with the acid phosphate-KCl of Guba and Straub (1943), precipitation in "crystals" by dilution, redissolution, precipitation of the actomyosin present with ATP, and reprecipitation of the myosin by dilution.

Myosin is very sensitive to the action of heavy metals to which it has a great affinity. Chemicals of high purity and glass-distilled water have to be used throughout. The temperature has to be kept low, as far as possible.

In earlier days the actomyosin present was eliminated by a rather complex process (I). Since ATP is available on the market now, these earlier methods can be supplanted by the superprecipitation of actomyosin.

Myosin may also be liberated from actomyosin by diluting the KCl presents to 0.3 *M* and spinning out the solution in the high-speed centrifuge. The actomyosin precipitates while myosin stays in solution. This method has been successfully used for some time in the author's laboratory and is also advocated by H. H. Weber and his associates who dilute to an ionic strength 0.3.

The detailed description of the single steps is as follows. The rabbit is killed by decapitation, quickly skinned and eviscerated, and dipped for 2 to 3 minutes in ice water. The muscles are excised, covered with ice until collected, and are then minced in a precooled meat grinder with small holes (2 mm).

The mince is suspended in 0°C KCl phosphate, containing 0.3 *M* KCl and 0.15 *M* potassium phosphate of pH 6.5, 300 ml being used for every 100 g of minced muscle. The subsequent data refer to this quantity of material.

The suspension is gently stirred with a glass rod for 10 minutes, while the temperature is kept at 0°C. Then 1200 ml of distilled water of 0°C is added, and the suspension is strained through cheesecloth. The residue is gently pressed out and can be used for the preparation of actin. The fluid is filtered through a Büchner funnel. Instead of paper, a cloth is used, covered with a thick sheet of fine paper pulp.[1] Two liters of ice-cold water is added slowly, while promoting parallel arrangement of micells and therewith promoting crystallization by stirring strongly. A silky sheen appears, indicating a regular arrangement and association of particles. The sheen gradually becomes stronger. Eventually the myosin precipitates in the form of microscopic needles. The suspension is set aside at 0°C for a few hours to give time for the precipitate to settle. Then the supernatant fluid is sucked off and the sediment is centrifuged, if possible at 0°C.

The pellet is dissolved in an equal volume of 0°C 1.2 *M* KCl solution, and the solution is diluted to a final volume of 100 ml by the addition of 0.6 *M* KCl.

Samples of 1 ml are taken, diluted with 3 ml water at room temperature. Varying amounts of 1% neutral ATP solution are added to find out what the smallest quantity would be which would cause maximum precipitation of the actomyosin present, forming a fine granular precipitate on the addition of ATP. Then the main fraction is likewise diluted by adding three volumes of water of room tempera-

[1] Paper pulp of sufficient dispersity can be prepared by suspending filter paper in water and treating it in the Waring Blendor for a few minutes and washing it thoroughly. Protracted treatment in the Blendor causes formation of colloidal cellulose.

ture. The corresponding amount (about 50 mg) of ATP is added, and the superprecipitated actomyosin is separated in the centrifuge. Since the superprecipitation at pH 6.5 is not reverted by cooling, this centrifugation can be done in the refrigerated centrifuge.

The supernatant fluid is diluted with double the amount of water at 0°C, and the precipitated myosin is allowed to settled for a few hours in the ice box; after decantation of the water, the myosin is separated in the centrifuge and dissolved in 0.6 *M* KCl or any other salt solution demanded by the experiment. If dissolved in 0.6 *M* KCl, neutralized and mixed with equal amounts of glycerol, it can be kept at —20°C apparently unchanged for weeks. This makes it possible to do a long series of experiments with the same preparation.

If the presence of small amounts of actin does not matter, the ATP treatment and the second precipitation can be omitted. If superprecipitated under conditions described above, the actin takes down a relatively large quantity of myosin.

The procedure described gives reliable results with rabbit muscle but does not necessarily work with other material.

Since the viscosity of the myosin, dissolved at pH 7 in 0.6 *M* KCl, is approximately normal, the concentration of such a myosin solution can be measured with a viscosimeter [Fig. 24(*a*)]. Small quantities of actin may greatly modify the viscosity, and, if such are present, ATP has to be added in small quantities to cause dissociation of the highly viscous actomyosin. ATP may slightly increase the viscosity of actin-free myosin if this was not completely solvatized.

Actin

The preparation of actin was worked out by Straub (1942–43) and Feuer, Molnár, Pettko, and Straub. The most advantageous starting material is rabbit muscle, extracted with the acid KCl phosphate, described in the preparation of myosin. It is essential that the temperature should be kept close to 0°C during the process of extracting myosin.

"The remaining muscle tissue [the muscle extracted with KCl phosphate, diluted and pressed out] is suspended in 5 volumes of a 0.4% $NaHCO_3$ solution of 22–25°C and kept at this temperature with constant stirring for 30 minutes. This fluid is again removed through a cheesecloth. The residue is suspended in one volume of a cold solution containing 0.01 *M* $NaHCO_3$ and 0.01 *M* Na_2CO_3. The temperature should be kept below 10°C. The mixture is thoroughly stirred. After 10 minutes the mixture is diluted with 10 volumes of glass-distilled water of 22–25°C and the fluid is squeezed through a cheesecloth as fast as possible. The residue is weighed, and for every 100 g of it 300

ml acetone are added. Temperature 22–25°C. Having stirred it for 10 minutes, the acetone is gently pressed out through a cheesecloth and one-third of the former amount of acetone is added again, stirred for 10 minutes, pressed down and the residue is dried in air at room temperature.

"After 5 to 10 hours of drying, actin may be extracted from the dry muscle. One gram of dry powder is treated with 20 volumes of CO_2-free glass-distilled water for 15 to 20 minutes at room temperature. The solution of actin is sucked off through a Büchner funnel. It contains 4–8 mg actin per milliliter.

"The dried muscle preparation is stable at least a week, it keeps well at a low temperature in a desiccator.

"*Notes.* It is most important to observe the prescribed temperature during each of the operations. Washing the muscle residue with alkaline solution leads to its moderate swelling. In these cases the fluid is pressed out only until it runs out smoothly under moderate pressure and it does not become turbid or viscous. The acetone should be cooled below 20°C, otherwise the mixture becomes too warm. We have found that the yield of the actin is increased if 0.0001 *M* $CaCl_2$ is added to the washing fluids in steps 3 and 4.[2] Addition of the same amount of $MgCl_2$ decreases the yield.

"The actin solutions thus obtained are usually more or less cloudy, depending on the state of nutrition of the animal. The cloudiness results from the presence of lipins, which were not removed by the acetone treatment. Repeated extraction of the dry muscle with acetone will remove them. This is best accomplished in a *Soxhlet* apparatus, if solid granulated $CaCl_2$ is added to the extracting acetone to fix the residual humidity. The dried muscle can be refluxed with boiling acetone more than ten times without destroying its actin content. The actin solutions obtained from exhaustively extracted dried muscle are completely limpid and colorless.

"If a salt-free actin solution is diluted with ten volumes of acetone and then a few drops of an acetate buffer [pH 4.7] are added, the actin is precipitated, while the lipid impurities remain in solution. Dissolving the precipitate in water, a concentrated solution of globular actin is obtained."[3]

A modification of this method was worked out by Guba and the author (I). In this modified method the muscle extracted with the acid phosphate, diluted and pressed out, is suspended in twenty volumes

[2] The two initial treatments of the KCl-phosphate-extracted muscle with alkaline water.

[3] Quotation from Feuer, Molnár, Pettko, and Straub.

of ice-cold 0.4% $NaHCO_3$, stirred for 20 minutes, separated by filtration through cheesecloth, and then suspended once more in the same amount of 0.4% $NaHCO_3$ at room temperature. After 20 minutes' stirring the muscle is separated again on the cheesecloth, pressed out by hand, and then put through the meat mincer. The muscle leaves the mincer in the form of threads. Without pressing these together the mass is transferred into the deep-freezer and kept there overnight at −20°C. The next day the mince is allowed to thaw and is then extracted with 10 volumes of 0° distilled water, pressed out and treated with acetone as described by Straub. It was found convenient to separate the muscle from the acetone in the centrifuge. In this way the acetone can be removed thoroughly without pressing the muscle into a lump. The residue is dried in air, placed into the Soxhlet and extracted as described by Straub, allowing the acetone to cover the material three times. The imbibed acetone is separated in the centrifuge, the material dried in air, and reduced to a fine powder in a mill. The grinding is necessary because the granulation of the material is rougher than that of muscle prepared according to Straub's original method.

The actin is extracted by covering 1 g of dried muscle with 20–30 ml CO_2-free water. The material swells and is allowed to stand without stirring for 20 minutes. Then it is poured through a Büchner funnel, where part of the water is filtered without suction. Finally a gentle suction is applied.

The transformation of G- into F-actin happens by adjusting the pH to 7 and adding 0.1 *M* KCl and 0.0005 *M* $MgCl_2$.

The concentration of an F-actin solution can be estimated approximately from its viscosity. The viscosity is only an apparent one, being anomalous and being complicated by thixotropy. An Oswald viscosimeter may yield useful results if relative values are wanted, as is often the case when the G-F transformation is followed. If more absolute values are wanted, it is advisable to work under higher pressure, let us say 60 mm Hg, as was done by Straub *et al.* In this case the error caused by slight variations in pressure will be smaller and thixotropy will have less effect. At this pressure at 24°C Straub *et al.* found up to η sp 1.

$$\frac{\eta \text{ sp}}{\text{mg N/ml}} = 1.7.$$

Special tricks have to be applied if we want to estimate the total actin content of a piece of muscle. Faced with such a problem, Balenovic and Straub ground up the tissue to a fine pulp with sand, suspended in it an excess of a solution of myosin in 0.6 *M* KCl, poor

in actin, and measured the drop in viscosity caused by ATP and calculated the actin content from it. (See next section.)

When G- and F-actin had to be estimated side by side, Straub took advantage of the fact that in 0.6 *M* KCl myosin inhibits the polymerization of actin. Part of the actin solution was thus added to the myosin unchanged, while another part was added after the whole actin was transformed into its F-form. The F-actin content of both fractions was then calculated from the drop in viscosity induced by ATP.

Actomyosin—Myosin B

F-Actomyosin can be prepared by mixing F-actin and myosin in the presence of 0.5 *M* KCl, or by mixing G-actin and myosin in the presence of 0.1 *M* KCl and traces of Mg. It can also be prepared directly from muscle as such, by prolonged extraction. If the muscles of the freshly killed rabbit are minced in a mincer with small holes (2 mm in diameter or less) and suspended in Weber-Edsall solution (0.6 *M* KCl, 0.01 *M* Na_2CO_3, 0.04 *M* $NaHCO_3$) using 3 ml of this fluid for every gram of muscle, and the suspension is placed in the refrigerator, it is usually found the next day to have turned into a thick, viscous fluid or a thixotropic gel. It is mostly too viscous to allow separation of the muscle particles in the centrifuge and has to be diluted somewhat with 0.6 *M* KCl for this purpose. If freshly minced muscle is suspended in 0.6 *M* KCl, the actomyosin present dissociates under the influence of the ATP present into actin and myosin, and the latter dissolves. In a few hours' time the ATP is decomposed, and the myosin again joins with the actin filaments to form actomyosin. The actin filaments break up into fragments which are taken into the solution by the excess of myosin. Such natural actomyosin thus consisting of fragments of the actin filament and myosin was called "myosin B." It mostly contains 1 part of actin for 5 to 6 parts of myosin. It can be purified by precipitation. If the solution is diluted with five volumes of water and the KCl concentration is lowered to 0.1 *M*, the actomyosin precipitates in the form of a voluminous, flocculent precipitate. The pellet usually contains 97 per cent water. Denser precipitates can be obtained by adding ATP, which turns precipitation into superprecipitation. If dissolved in strong 0.6 *M* KCl, there is no difference between actomyosin which has been superprecipitated or merely precipitated.

Actomyosin has a high and anomalous viscosity. Dissolved in 0.5 or 0.6 *M* KCl at pH 7 (veronal acetate buffer), its specific viscosity rises very fast if its concentration is increased above 0.15% (Fig. 20). If ATP is added in small quantities (1–2 drops or 1% ATP for 5 ml),

the actomyosin dissociates and the viscosity drops to the additive value of the actin and myosin present.[4]

The specific viscosity of actomyosin and accordingly its drop, observed when ATP is added, depend on the relative proportion of actin and myosin present. This drop, earlier called "activity," was found by Balenovic and Straub to be the greatest if actin and myosin were present in a relation of 1:2.5 which corresponds to the relation also found in muscle. The two curves in Fig. 20 illustrate the situation and help to calculate relative actin and myosin content from the drop in viscosity. With 1 mg myosin and 0.2 mg F-actin present per milliliter of fluid, the addition of ATP causes the specific viscosity to fall to one-half its original value. If very small quantities of actin are present, the test can be made more sensitive by decreasing the pH and salt concentration (Straub, 1941–42).

The log of the relative viscosity of actomyosin being a linear function of concentration in a wide range, it is advantageous to calculate, with the logs of viscosities instead of the viscosities themselves.

When trying to estimate the actin content of actomyosin, it is as well to collect some experience about the behavior of actin and myosin first by preparing these substances and mixing them and establishing empirical curves which can help to evaluate results.

Actomyosin Threads

As described by H. H. Weber, threads of actomyosin can be prepared by squirting the salt solution of "myosin" (actomyosin) in a thin jet into distilled water. The simple procedure is shown in Fig. 55. The actomyosin dissolved in 0.6 *M* KCl is sucked up into a glass tube one end of which is pulled out, the end narrowing rather suddenly to a capillary. The top end is connected with rubber tube. Blowing gently into this tube will make the viscous actomyosin solution flow out. The capillary end is dipped into water and moved back and forth at a uniform speed. The actomyosin, losing its salt, gelatinizes to a thread which settles to the bottom. The thickness of the threads can be adjusted by gradually breaking off the narrow end of the capillary, thus widening the aperture. The water in the basin should be at least 5 cm deep to give time for the thread to solidify before reaching the bottom.

It is essential to use glass-distilled water containing no trace of heavy metal. Common distilled water usually is only a dilute copper solution. Myosin has a great affinity to heavy metals and a small

[4] As pointed out by Weber (1950), it is the log of the viscosities which add up and not the viscosities themselves. The difference is usually negligible.

amount of actomyosin will pick out the traces of copper in the relatively large volume of water. Threads prepared without precaution may even turn green owing to the adsorbed copper. Such threads will consist of denatured actomyosin, as revealed by their elasticity, allowing elastic extension by 100–300 per cent. Native actomyosin threads can be stretched only a few per cent, and then they break.

If the salt is washed out completely, the threads swell to a loose glassy mass, especially if the actomyosin solution is alkaline. To prevent such swelling it is advisable to use a dilute (0.05 *M*) KCl solution instead of water. The addition of some Mg (0.0005–0.001 *M*) is also advisable, Mg being an essential constituent of the system and promoting gelation.

FIG. 55. The preparation of actomyosin threads.

Immediately after their preparation the threads are soft and fragile. It is advisable to store them for a few hours before use, during which time hysteresis makes them more resistant mechanically and reduces their tendency to dissolve and dissociate, without interfering with contractility.

In 0.1 *M* KCl the threads "contract" when ATP is added. If the salt concentration is raised (in the presence of ATP), the thread tends to swell again to its original volume and dissolve; 0.001 *M* Mg renders actomyosin more insoluble and increases the affinity to ATP, so in its presence the "contracted" threads can be made to swell to their initial dimensions again, without dissolution, by raising the KCl concentration from 0.1 *M* to about 0.25 *M* in the presence of ATP. Threads about

0.2 mm in diameter and 2–3 mm in length are the most convenient. Their contraction is best observed under the microscope at low power. Threads can be transferred on the object slide by sucking them up in a pipet or by means of a strip of celluloid, bent at one end and used as a spatula. Actomyosin easily sticks to glass but not to celluloid.

If contraction is studied quantitatively, it is necessary to have threads of uniform diameter, prepared mechanically under standardized conditions. Such mechanical devices have been described by Gerendás and by Buchthal and his associates (1947). The former makes the water move by rotating the circular basin, the latter moves the capillary.

Gerendás prepared threads with orientated micells by stretching them after partial denaturation, achieved by placing them into 0.01 *M* $ZnSO_4$ solution for 5 minutes. This made the thread extensible by 200 per cent, without destroying "contractility." If the thread is kept in an extended condition for a few minutes, it sets and no longer contracts on release. This method is fairly sensitive, and one may have difficulty in finding the borderline between complete and partial denaturation.

More reproducible is the method of Buchthal *et al.* (1947) who dried threads at constant length. The drying should be rather superficial. If dried too long, for more than an hour, threads lose their contractility. Threads can be dried with fixed ends by placing them across a corresponding split (about 2 mm wide) cut into hardened filter paper. Drying can be watched under the microscope by observing the diameter. As soon as this does not appreciably decrease any farther, the theads are ready for use.

If the ATP acts very abruptly, it may make the superficial layers of a thread contract before the core can shrink. The result is that the outer layer breaks up like a crocodile skin. If the thread contains much free myosin, the actomyosin may contract, forming a solid core inside a non-contracting mass. If the thread contains smaller amounts of free myosin and the ATP contraction is watched in a dark-field illumination, one may find contraction accompanied by the thread pressing out a cloud of collodial matter.

Superprecipitation

Superprecipitated actomysin consists of a rough suspension of particles very different in size. Consequently the precipitate will scatter light under a low angle. There is no increased light adsorption. It follows that if the superprecipitation has to be measured quantitatively by means of a photoelectric cell, we have to place the photoelectric

cell directly in the path of the main beam and measure the light lost, or we have to place our photoelectric cell just outside the primary beam and measure the light scattered. Turbidity meters which collect light from a wider range are unfit for this purpose.

The intensity of superprecipitation can also be measured roughly by measuring the volume of the sediment and the speed of sedimentation.

In direct visual observation superprecipitation is a very striking phenomenon if conditions are adapted to the specific conditions of the low-angle scattering. First of all, the reflections on the outer surface of our test tubes must be excluded. This can be done by immersing the test tube in a square water bath with parallel glass walls. Since the refractive index between water and glass is smaller than between air and glass, this eliminates most reflections.

We can produce a simple low-angle illumination in two ways. The author usually sticks 0.6-cm-wide strips of black tape horizontally to the back wall of the water bath at distances of 0.6 cm from one another and illuminates the tubes through the rough grid thus formed from behind with roughly parallel lights (incandescent tube). The superprecipitation is now observed from the front against the alternating strips of black and white. S. S. Spicer (personal communication) prefers to stick one wide tape to the back wall of the water bath, forming a uniform background at the level at which the test tubes contain the observed suspensions. The light is sent in from above and below this wide tape.

The sequence in which actin, myosin, salts, and ATP are mixed is not immaterial. If we want to see "contraction" in our solution, as shown in Fig. 23(*d*), it is best to dilute the KCl solution of myosin until it contains 0.1 *M* KCl and the proper amount of myosin (0.2–0.5%) and a trace of Mg (0.0005 *M*), add ATP (0.02–0.05%), and add the F-actin solution last, and mix the two fluids by turning over the tube once. The ATP added to the myosin increases its dispersity. If the actomyosin is repeatedly shaken, the continuity of the actomyosin network is broken up and a rough floccular precipitate will be obtained. In order to produce superprecipitation of G-actomyosin we add ATP to myosin first and G-actomyosin after. If G-actomyosin is added first to the actin and ATP after, F-actomyosin is obtained.

Under proper illumination the superprecipitation is a very striking phenomenon. Seeing it in the practical course, side by side with contracting actomyosin threads and glycerinated psoas fibers, will leave little doubt in the student's mind of the close relation between these phenomena.

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